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High-Throughput Data Analysis: Application to Micronuclei Frequency and T-cell Receptor Sequencing

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High-Throughput Data Analysis: Application to Micronuclei Frequency and T-cell Receptor Sequencing

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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The advent of high-throughput sequencing has brought about the creation of an unprecedented amount of research data. Analytical methodology has not been able to keep pace with the plethora of data being produced. Two assays, ImmunoSEQ and the cytokinesis-block micronucleus (CBMN), that both produce count data and have few methods available to analyze them are considered .

ImmunoSEQ is a sequencing assay that measures the β T-cell receptor (TCR) repertoire. The ImmunoSEQ assay was used to describe the TCR repertoires of patients' that have undergone hematopoietic stem cell transplantion (HSCT). Several different methods for spectratype analysis were extended to the TCR sequencing setting then applied to these data to demonstrate different ways the data set can be analyzed. The different methods include CDR3 distribution perturbation, Oligoscores, Simpson's diversity, Shannon diversity, Kullback-Leibler divergence, a non-parametric method and a proportion logit transformation method. Herein we also demonstrate adapting compositional data analysis methods to the TCR sequencing setting. The various methods were compared when analyzing a set of 13 subjects who underwent hematopoietic stem cell transplantation. The eight subjects who developed graft versus host disease were compared to the five who did not. There was no little overlap in the results of the different methods showing that researchers must choose the appropriate method for their research question of interest.

The CBMN assay measures the rate of micronuclei (MN) formation in a sample of cells and can be paired with gene expression or methylation assays to determine association between MN formation and other genetic markers. Herein we extended the generalized monotone incremental forward stagewise (GMIFS) method to the situation where the response is count data and there are more independent variables than there are samples. Our Poisson GMIFS method was compared to a popular alternative, `glmpath`, by using simulations and applying both to real data. Simulations showed that both methods perform similarly in accurately choosing truly significant variables. However, `glmpath` appears to overfit compared to our GMIFS method. Finally, when both methods were applied to two data sets GMIFS appeared to be more stable than `glmpath`.

1 T-Cell Receptors and the Adaptive Immune System

1.1 T-cell Biology

The adaptive immune system is responsible for recognizing foreign and self antigens and mounting the proper response. This is known as acquired immunity because the body "remembers" the specific antigen and can mount a quicker and more potent response each subsequent time the body encounters that antigen. T and B cells are responsible for maintaining an acquired immunity. T cells are a type of cell that maintains the body's immunity by activating genes that create cell surface receptors that recognize foreign and self antigens. These genes and subsequently receptor proteins are passed on to future generations of cells during cell division. T-cell receptors (TCR) are heterodimers that contain an α and a β chain or a γ and a δ chain. The chains are made up of several different regions; a constant region, a variable region, a transmembrane domain and a short cytoplasmic tail. Further, the variable region of the β chain of TCR's is made up of the variable (V), joining (J) and diversity (D) gene segments [Robins et al., 2009, Rudolph et al., 2006].

T-cells are activated when the TCR recognizes an antigen bound to a major histocompatibility complex (MHC) molecule presented to it by an antigen presenting cell (APC) [Avent, 2012]. Once the antigen is recognized an immune response is launched. The first step in a memory response is to have a TCR that recognizes a specific antigen. Because there is a large diversity of antigens the human body can come in contact with, the immune system must have a way to generate a large diversity of TCR's. Producing a large diversity of TCR's is accomplished by a process called VDJ recombination (Figure 1). VDJ recombination is the joining of different gene segments to create unique amino acid sequences. Specifically, V, D and J segments that are spread across human chromosomes 2,14 and 22 are combined to make a unique VDJ combination.

In this review we are only interested in VDJ recombination of the β chain of TCR's (TCRβ). There are 52 known V gene segments, 2 D gene segments and 13 J gene segments

[Janeway et al., 2001] in the TCRβ chain. All possible VDJ combinations yield $13 \times 2 \times 52 = 1352$ possible combinations. This is not a large variety and further diversity in TCR's is added by the amino acid sequence encoded in the third complementary-determining region (CDR3) [Robins et al., 2009]. The CDR3 region occurs at the VDJ junction. As can be seen in Figure 2, this junction includes part of the V segment, all of the D segment and the J segment. The CDR3 region undergoes template-independent additions and deletions of nucleotides during TCR gene rearrangement. This greatly increases TCRβ diversity. Arstila et al. [1999] reported estimates of 10^6 unique TCRβ CDR3 sequences per person. By measuring the abundance of each clone a better understanding of the human immune response can be gained [Carlson et al., 2013].

Figure 1: VDJ Recombination: A schematic of how separate V,D and J segments are recombined into a functional TCR protein (Adapted from Janeway et al. [2001]).

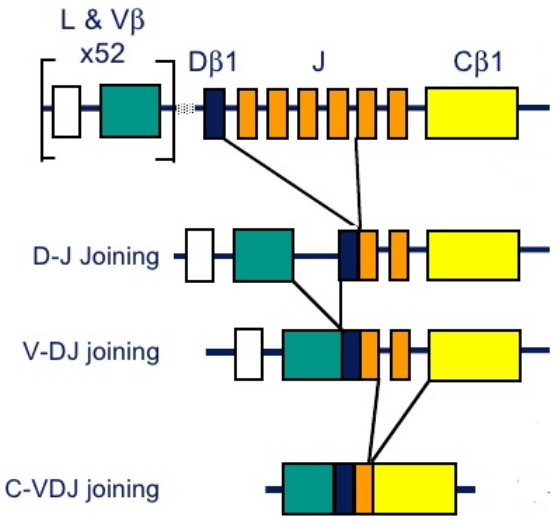
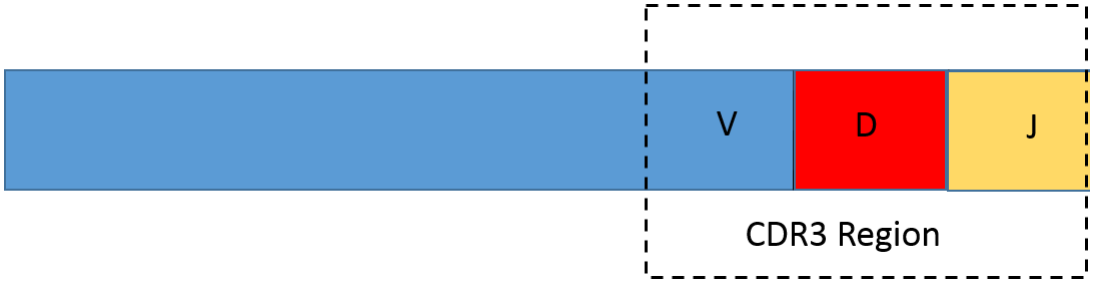


Figure 2: CDR3 Region: A diagram of where the CDR3 region occurs in a recombined VDJ segment.



1.2 Technology for Measuring TCRβ Clones

Historically, measuring TCR abundance was done by spectratype analysis, a process that measures relative abundance of TCR clones. Unfortunately, spectratyping can only distinguish a particular V, D or J segment. For a specific V or J gene the clones are separated by size rather than sequence [Kepler et al., 2005]. This is useful in general terms to monitor TCR diversity. A more detailed review of spectratype analysis methodology can be found in section 1.6.

Figure 4 shows a schematic of how spectratyping assay is done. First a sample is collected and the T-cells are isolated. PCR is then used to replicate only a specific TCRβ gene segment (V, D or J) by using primers specific to a TCRβ segment. The mixture of CDR3 region clones is then separated by size using electrophoresis. The results are then presented on histograms with number of TCR's at each specific size [Kepler et al., 2005]. Note that spectratyping separates CDR3 regions by size, if two different CDR3 sequences are of the same length they will be counted in the same bin. Figure 3 shows examples of some common CDR3 length distributions that result from spectratype analysis.

Recent improvements in sequencing technology allow for much greater detail in measuring TCR diversity [Robins et al., 2009]. Adaptive Biotechnologies developed an immune profiling assay, ImmunoSEQ, that uses parallel sequencing to capture 48 possible V segments, 13 J segments and 2 D segments by simultaneous use of PCR primers for each segment [Robins et al., 2009]. This allows for simultaneous sequencing of a large portion of the human T-cell

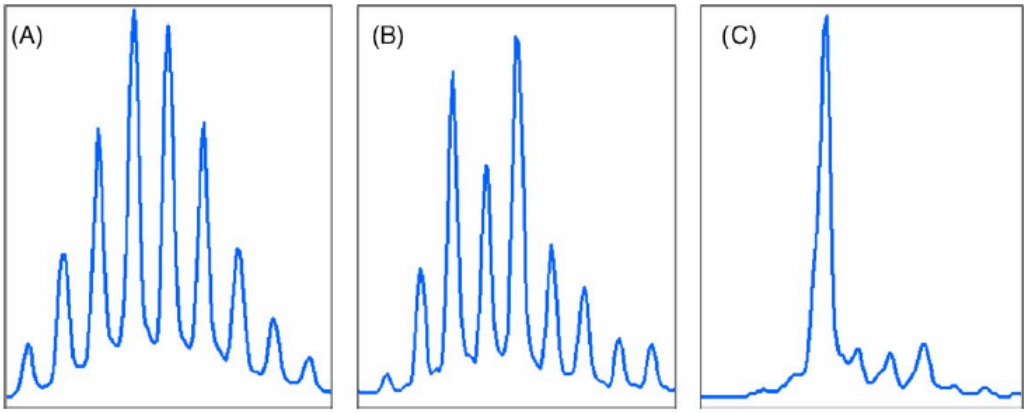


Figure 3: TCR distributions: (A) Shows a typical Gaussian polyclonal distribution. (B) Shows a non-Gaussian distribution that is also polyclonal. (C) shows a non-Gaussian distribution that is monoclonal [Miqueu et al., 2007] with permission.

reportoire. The assay maximizes throughput by only amplifying and sequencing the region of the TCR that contains the CDR3 region. This allows for short reads, 60 nucleotides in length, to be used to identify each sequence. Further, the assay can be used on both cDNA and genomic DNA [Robins et al., 2009]. Recently, Adaptive Biotechnologies developed a new method to correct for PCR bias caused by using so many different primers [Carlson et al., 2013]. By using sequencing, researchers can distinguish TCRβ clones down to the level of unique CDR3 clones.

Figure 4: **Spectratype Schematic** Shows steps taken to run to generate spectratype data. (A) shows using electrophoresis to separate the mRNA by size. (B) shows measuring the band intensity. (C and D) show translating the band intensities into a distribution by size [Miqueu et al., 2007] with permission.

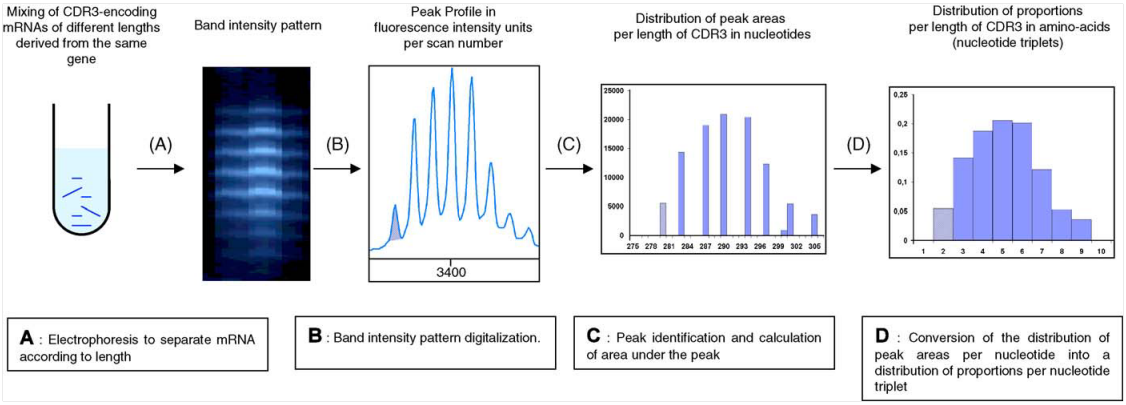
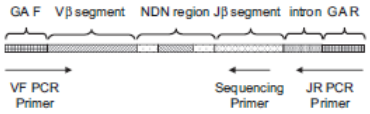


Figure 5 shows a schematic of a generic PCR product used by the ImmunoSEQ assay. 13 reverse J primers and 48 forward V primers were designed to capture $13 \times 2 \times 48 = 1248$ possible VDJ combinations. All sequences contain small portions of the V and J segments as well as the CDR3 region. This allows not only for gene segment family identification but also unique CDR3 identification.

Figure 5: **Sequencing Assay Strategy Schmatic** A generic TCRβ CDR3 PCR product is shown. Primers include parts of the V and J segments and also the Genome Analyzer Cluster station (GA F and GA R). The NDN region represents the CDR3 region. The primers were designed to capture just enough of the J and V segments to allow identification [Robins et al., 2012] with permission.



1.3 ImmunoSEQ Precision, Accuracy and Sensitivity

Robins et al. [2012] conducted experiments using the ImmunoSEQ platform to determine its precision, accuracy and specificity. Samples containing spiked-in clones of known concentration ranging from 10 to 100,000 cells per million were created. The experiments show that the assay can detect clones reliably at a level of one cell per 100,000.

The spike-in experiments were designed to test the assay's accuracy and sensitivity. Different mixtures of five CD4+ T-cell clones specific for GAD65 were spiked into a background of one million CD4+ T cells. Three different mixtures were made using these five clones. Two other T-cell clones that targeted tumor-associated NY-ESO-1 antigen were also used. These were spiked directly into autologous peripheral blood mononuclear cells instead of separated cells. The titrations that were used were 1/100,000, 1/10,000, 1/1000 and 1/100. The different mixtures were then sequenced using the method described in section 1.2. The raw data results in counts for each unique CDR3 region. Finally, a nearest neighbor alogrithm was used to merge closely related sequences to remove PCR and sequencing errors. The pro-

cessed data were then used to estimate the frequencies of each spiked in clone. The results show that 6 out of 7 of the clones had an observed frequency very close to the expected. The 7th clone was not detected at all because it contained a 16 nucleotide deletion and the assay is designed to capture all clones with deletions of 14 nucleotides or fewer (this deletion length accounts for 99% of TCRβ's) [Robins et al., 2012]. Thus, the assay is extremely accurate in detecting clones even at low concentrations.

Finally, the reproducibility of the assay was tested. Blood was drawn from one subject and DNA was extracted two independent times (DNA_A and DNA_B). The DNA_A sample was then PCR amplified two independent times (PCR_A1 and PCR_A2) while the DNA_B sample was only PCR amplified once (PCR_B1). Finally, sample PCR_A1 was sequenced two independent times (SeqA1a and SegA1b) while samples PCR_A2 and PCR_B1 were only sequenced once (SegA2a and SeqB1a). Robins et al. [2012] showed that sequencing from the the same PCR library is highly reproducible. Robins et al. [2012] showed that sequencing from different PCR libraries is also reproducible but not quite as much. Finally, Robins et al. [2012] showed that sequencing different DNA extractions is also reproducible but again not quite as much as the other two conditions. In the different blood draw samples, approximately 55 % of all reads were obtained from shared sequences. This is probably due to a blood draw being a finite sample so each one is a random subset of all clones [Robins et al., 2012].

1.4 Unseen Species and Estimating the TCRβ Repertoire using ImmunoSEQ data

1.4.1 Unseen Species Problem

The unseen species problem is one that has been tackled several times over the years. Efron and Thisted [1976] described a method for estimating unseen species. The unseen species problem was first considered in ecology, where researchers needed to estimate the number

of different types of species in a given area assuming they could not sample every organism. That is, if a given area is sampled and n species are found, the unseen species problem is to estimate how many more unique species are in the area that were missed by the sample. A different application that may help elucidate this concept is to consider William Shakespeare's vocabulary. Given all the different words he used in his writing, we may want to estimate how many words Shakespeare actually knew assuming he did not use all of them in his works. The unseen species estimation problem in this case is, if a new work from Shakespeare is found, to estimate the number of new words not used in his previous works. Shakespeare's works are comprised of 884,647 total words, 31,534 of which are unique [Efron and Thisted, 1976]. Let n_x represent the number of words occuring x times (x= 1 to ∞). The method described by Efron and Thisted [1976] says, assuming the new works are the same size as the old, 11,460 new words would be expected. The basic model uses an empirical Bayes approach and is described later in this section. Recently, this method has been modified to genome sequencing [Ionita-Laza et al., 2009] and T-cell receptor sequencing [Robins et al., 2009].

The methodology described by Efron and Thisted [1976] can be thought about using species trapping terminology. Suppose there exist S species and after trapping for one unit of time we capture x_s members of species s. We then assume that x_s has a Poisson distribution with a mean of λ_s. It is convenient to consider the time period to be $[-1, 0]$ and we wish to extrapolate to a time t in the future. Let $x_s(t)$ be the number of times species s appears in $[-1, t]$.

Before moving on, it is of relevance to review the Poisson distribution. The Poisson distribution is a widely used discrete distribution for modeling count data. For example, the Poisson distribution can be used to model the number of occurences of a phenomenon in a given time period [Casella and Berger, 2002]. In this case, the number of species seen in a given area in a given time period. The basic assumption the Poisson distribution is built on is that the probability of occurence is proportional to length of waiting time. So the Poisson

distribution has one parameter λ, sometimes called the intensity parameter [Casella and Berger, 2002]. A random variable, X has a Poisson(λ) distribution if

$$P(X = x|\lambda) = \frac{e^{-\lambda}\lambda^x}{x!}, x = 0, 1, 2, ...$$

where the $\mathbb{E}(X) = \lambda$ and $\text{var}(X) = \lambda$. Two useful characteristics of the Poisson distribution are that the sum of independent Poisson random variables is itself Poisson distributed and a Poisson random variable conditioned on a sum of Poisson variables is binomial. More formally,

Theorem 1. *If $X \sim Poisson(\theta)$ and $Y \sim Poisson(\lambda)$ and X and Y are independent, then $X + Y \sim Poisson(\theta + \lambda)$ [Casella and Berger, 2002].*

The Poisson distribution assumption implies, using Theorem 1, that $x_s(t)$ is distributed Poisson(λ_s(1+t)). Furthermore, we can say that

$$x_s|x_s(t) \sim \text{Binomial}\left(x_s(t), \frac{1}{1+t}\right)$$

.

Proof. Assuming

$$x_s \sim Poisson\left(\lambda_s\right)$$

Then

$$x_s(t) \sim Poisson(\lambda_s(1 + t))$$

given there are $1 + t$ time points in the $[-1, t]$ and by Theorem 1. Next to show $x_s|x_s(t)$ is binomially distributed, let $x_s(t) = x_s + x_s(t-1)$ where $x_s(t-1)$ does not include the interval $[-1, 0]$. Note that $\Pr(x_s(t-1) = x_s(t) - x_s \mid x_s) = \Pr(x_s(t-1) = x_s(t) - x_s)$ because $x_s(t-1)$ does not include the interval $[-1, 0]$ and therefore is not dependent on x_s. So we have

$$
\begin{aligned}
\Pr(x_s \mid x_s(t)) &= \frac{\Pr(x_s, x_s(t) = x_s + x_s(t-1))}{\Pr(x_s(t) = x_s + x_s(t-1))} \\
&= \frac{\Pr(x_s)\Pr(x_s(t-1) = x_s(t) - x_s \mid x_s)}{\Pr(x_s(t) = x_s + x_s(t-1))} \\
&= \frac{\Pr(x_s)\Pr(x_s(t-1) = x_s(t) - x_s)}{\Pr(x_s(t) = x_s + x_s(t-1))} \\
&= \frac{\exp(-\lambda_s)\lambda_s^{x_s}}{x_s!} \times \frac{\exp(-\lambda_s t)(\lambda_s t)^{x_s(t-1)}}{x_s(t-1)!} \times \frac{x_s(t)!}{\exp(-\lambda_s(1+t))(\lambda_s(1+t))^{x_s(t)}} \\
&= \binom{x_s(t)}{x_s} \frac{\exp(-\lambda_s)\lambda_s^{x_s}\exp(-\lambda_s t)(\lambda_s t)^{x_s(t)-x_s}}{\exp(-\lambda_s(1+t))(\lambda_s(1+t))^{x_s(t)}} \\
&= \binom{x_s(t)}{x_s} \frac{\lambda_s^{x_s}(\lambda_s t)^{x_s(t)-x_s}}{(\lambda_s(1+t))^{x_s(t)}} \\
&= \binom{x_s(t)}{x_s} \frac{\lambda_s^{x_s}(\lambda_s t)^{x_s(t)-x_s}}{(\lambda_s(1+t))^{x_s(t)-x_s+x_s}} \\
&= \binom{x_s(t)}{x_s} \left(\frac{1}{1+t}\right)^{x_s} \left(\frac{t}{1+t}\right)^{x_s(t)-x_s} \\
&= \binom{x_s(t)}{x_s} \left(\frac{1}{1+t}\right)^{x_s} \left(1 - \frac{1}{1+t}\right)^{x_s(t)-x_s}
\end{aligned}
$$

This is a binomial distribution with $n = x_s(t)$ and $p = \frac{1}{1+t}$. □

Let $G(\lambda)$ be the empirical cumulative distribution function of $\lambda_1, ..., \lambda_S$. If n_x is the number of species observed x times in $[-1, 0]$, let

$$\eta_x = E(n_x) = S \int_0^\infty (\frac{e^{-\lambda}\lambda^x}{x!}) dG(\lambda), \tag{1}$$

where λ is the λ associated with $G(\lambda)$. Next, let $\Delta(t)$ be the expected number of species in $(0, t]$ but not in $[-1, 0]$. Note that using equation 1 the probability of not seeing a species in a given interval (observing $x = 0$) is given by $\exp(-\lambda)$. Likewise, the probability of not seeing a species in the interval $[-1, 0]$ is $\exp(-\lambda)$ so that the probability of seeing a species in the interval $(0, t]$ is $1 - \exp(-\lambda t)$. Thus,

$$\Delta(t) = S \int_0^\infty \exp^{-\lambda}(1 - \exp^{-\lambda t}) dG(\lambda).$$

To estimate $\Delta(t)$ we can use a Taylor series expansion of $1 - \exp^{-\lambda t}$,

$$1 - \exp^{-\lambda t} = \lambda t - \frac{\lambda^2 t^2}{2!} + \frac{\lambda^3 t^3}{3!} - ...$$

and substituting the right hand expression into the expression for $\Delta(t)$ we get

$$\Delta(t) = S \int_0^\infty \exp^{-\lambda}(\lambda t - \frac{\lambda^2 t^2}{2!} + \frac{\lambda^3 t^3}{3!} - ...) dG(\lambda)$$

which can be rexpressed as

$$\Delta(t) = S \int_0^\infty \left(\frac{\exp^{-\lambda} \lambda t}{1!} - \frac{\exp^{-\lambda} \lambda^2 t^2}{2!} + \frac{\exp^{-\lambda} \lambda^3 t^3}{3!} - ... \right) dG(\lambda).$$

Substituting equation 1 into the right hand side we get

$$\Delta(t) = \eta_1 t - \eta_2 t^2 + \eta_3 t^3 - ...$$

Thus an estimate for $\Delta(t)$ is

$$\hat{\Delta}(t) = n_1 t - n_2 t^2 + n_3 t^3 - \dots.$$

This estimate works well when $t = 1$ but when $t > 1$ the t^x values cause wild oscillations which may cause convergence issues. Euler's transformation is one way to force the series to converge. Euler's transformation is defined [Knopp, 2013] as

$$\sum_{k=0}^{\infty} (-1)^k a_k = \sum_{n=0}^{\infty} \frac{\Delta^n a_0}{2^{n+1}}$$

where

$$\Delta^k a_0 = \sum_{m=0}^{k} (-1)^m \binom{k}{m} a_{k-m}$$

which is the form that the unseen species estimate takes. That is

$$\sum_{k=0}^{\infty} (-1)^k a_k = \sum_{x=1}^{\infty} (-1)^{x+1} \eta_x t^x.$$

Application of this transformation is discussed further in section 1.4.2.

1.4.2 TCRβ CDR3 Diversity

Robins et al. [2009] used the unseen species method described in section 1.4.1 to estimate the human TCRβ repertoire. Using the same terminology as in 1.4.1, the total number of unique TCRβ CDR3 sequences in the reportoire is represented by S. Let x_s represent the number of a specific CDR3 sequence, s, observed in an experiment. Further, assuming T-cells circulate freely in the blood, it can be assumed that each CDR3 count, x_s, is distributed $Poisson(\lambda_s)$. Let $x_s(t)$ represent the number of times a CDR3 sequence was observed in sequencing experiment t and every previous experiment. Here, t represents the numbered experiment, $1, 2, ...$, that was sequenced. Finally, let n_x represent the number of CDR3 sequences observed exactly x times. Once again, we need to estimate $\Delta(t)$, the number of CDR3 sequences expected to be observed in all sequencing experiments except the first one, $t = 1$. This gives us an estimate of the total number of unique CDR3 sequences that could be observed in one subject. Once again the estimate for $\Delta(t)$ is

$$\hat{\Delta}(t) = n_1 t - n_2 t^2 + n_3 t^3 - ...$$

By letting $t = \frac{u}{2-u}$ the following relationship is derived using Euler's transformation from section 1.4.1

$$\sum_{x=1}^{\infty}(-1)^{x+1}\eta_x t^x = \sum_{y=1}^{\infty}\xi_y u^y$$

where

$$\xi_y = \sum_{x=1}^{\infty}\binom{y-1}{x-1}\frac{(-1)^{x+1}}{2^y}\eta_x = \frac{1}{2^y}\delta^y(\eta_1)$$

and

$$\delta^0 = \eta_1, \delta^1 = \eta_1 - \eta_2, \delta^2 = \eta_1 - 2\eta_2 + \eta_3, \ldots.$$

Let

$$\Delta^{x_0}(t) = \sum_{x=1}^{\infty}(-1)^{x+1}\eta_x t^x$$

$$\Delta^{x_0}(u) = \sum_{y=1}^{\infty}\xi_y u^y$$

$$\Delta(t) = \lim_{x_0\to\infty}\Delta^{x_0}(t)$$

$$\Delta(u) = \lim_{x_0\to\infty}\Delta^{x_0}(u).$$

By definition $\Delta(t) = \Delta(u)$ if both limits exist. Here $\Delta^{x_0}(u)$ converges much more quickly to the common limit [Efron and Thisted, 1976]. We can estimate ξ_y by substituting n_x for η_x and then estimate $\Delta(t)$ using

$$\Delta^{x_0}(u) = \sum_{y=1}^{\infty}\xi_y u^y = \sum_{y=1}^{x_0}\frac{1}{2^y}\delta^y(\eta_1)u^y, u = \frac{2t}{1+t}.$$

Using the results from their sequencing experiments, Robins et al. [2009] estimated the human TCRβ repertoire to be 10^5 CDR3 sequences. This result is similar to that obtained by Arstila et al. [1999] who estimated it to be 10^6.

1.4.3 Models for Estimating Population T-cell Diversity

Section 1.6 describes several T-cell receptor analysis methods in detail. This section will briefly describe several other methods present in the literature. Specifically, models to estimate overall T-cell diversity rather than just sample T-cell diversity will be presented.

Sepúlveda et al. [2010] described a series of Poisson abundance models that can be used to estimate the diversity in the original T-cell population. The clonal size distribution can be described by the following Multinomial law

$$P\left[m_x \middle| D, \boldsymbol{\eta}\right] = \frac{D!}{(D-M)\prod_{x=1}^{n} m_x!} \left[p_{\boldsymbol{\eta}}(0)\right]^{D-M} \prod_{x=1}^{n} \left[p_{\boldsymbol{\eta}}(x)\right]^{m_x}, \tag{2}$$

where D is the number of distinct clonotypes in the population which is the population diversity in this case. m_x is the number of clonotypes with x copies in the sample and $p_{\boldsymbol{\eta}}(x)$ is the probabilty of a clonotype being sampled x times and is described by a model with parameter vector $\boldsymbol{\eta}$. M is the sample diversity and n is the sample size. By assuming $p_{\boldsymbol{\eta}}$ follows a Poisson distribution, one can obtain the class of Poisson abundance models.

The simplest Poisson abundance model is assuming a homogeneous Poisson distribution for all clonotypes. That is

$$p_\lambda(x) = \frac{e^{-\lambda}\lambda^x}{x!} \tag{3}$$

where λ is the sampling rate. This can then be plugged back into equation 2 for $p_{\boldsymbol{\eta}}$. However, it is very simplistic to assume all clonotypes have the same sampling rate. Thus, different distribution can be obtained by applying a distribution to λ.

The first model to consider is the Poisson-Gamma model where λ follows the Gamma distribution. This assumes clonotypes have different sampling rates based on their clonal size (the number of times a clonotype appears in the sample). By mixing equation 3 with a Gamma distribution for λ we get

$$p_{\alpha,\beta}(x) = \int_0^\infty \frac{e^{-\lambda}\lambda^x}{x!}\frac{\beta^\alpha \lambda^{\alpha-1}e^{-\lambda\beta}}{\Gamma(\alpha)}d\lambda = \frac{\Gamma(x+\alpha)}{\Gamma(x+1)\Gamma(\alpha)}\left(\frac{\beta}{\beta+1}\right)^\alpha\left(\frac{1}{\beta+1}\right)^x, \tag{4}$$

where α and β are the shape and scale parameters of the Gamma distribution.

Another model is the Poisson-lognormal model. This model assumes that λ follows a lognormal distribution with parameters μ and σ^2. Using this model the sampling probability is

$$p_{\mu,\sigma^2}(x) = \int_0^\infty \frac{e^{-\lambda}\lambda^i}{x!}\frac{e^{\frac{(\log\lambda-\mu)^2}{2\sigma^2}}}{\sqrt{2\pi\sigma^2}}d\lambda. \tag{5}$$

This does not have a closed form and a numerical algorithm can be used such as the Newton-Raphson to calculate the probabilities.

The final model assumes λ follows an exponential distribution with paramter $\mu = e^{-\omega}/(1-e^{-\omega})$. Further, ω follows an exponential distribution with parameter ρ. Thus we get the following sampling distribution

$$p_\rho(x) = \rho\frac{\Gamma(x+1)\Gamma(\rho+1)}{\Gamma(x+\rho+2)}. \tag{6}$$

Using the sampling probability equations, the diversity, D, can be estimated by maximizing equation 2. Rempala et al. [2011] and Greene et al. [2013] expand these models to the multivariate setting to estimate population diversity. To choose the model that best fits the data goodness of fit tests can be computed such as the Pearson chi-squared test and AIC measure. Once an appropriate model is chosen, reportoires can be compared. The diversities can be compared by tests such as the Chi-squared or Kolmogorov-Smirnov tests. Because these tests are very sensitive to sample size, Mehr et al. [2012] suggest using machine learning methods such as repoirtoire clustering by similarity.

1.5 Application of TCRβ Studies

1.5.1 Hematopoietic stem cell transplantation

Hematopoietic stem cell transplantation (HSCT) is used to treat hematological disorders such as blood cancers. The procedure involves replacing diseased host cells with healthy donor cells. A result of this procedure is graft-versus-tumor effect (GVT), which is when the reconstituted T-cells recognize the blood cancer and mount an immune response. Conversely, the reconstituted T-cells can recognize the new host and mount an autoimmune response known as graft-versus-host-disease (GVHD) [Avent, 2012]. Because GVHD is potentially lethal, it is extremely important to match donors to recipients to minimize the risk of donor T-cells recognizing the recipients cells. The ability to measure TCRβ clone abundance can greatly help in understanding the immune system's requirement for initiating GVT versus GVHD.

1.5.2 Case Studies

Several studies have used the ImmunoSEQ assay to describe the T-cell repertoire in a variety of circumstances such as monitoring minimal residual disease (MRD), measuring diversity pre- and post-treatment and describing the T-cell repertoire during viral infection. This section will briefly describe six studies that have utilized the ImmunoSEQ assay.

Weng et al. [2013] studied the ability of high-throughput sequencing to monitor MRD in T-cell lymphoma patients. Samples from 10 patients were examined using the ImmunoSEQ assay. Tumor clones were identified prior to HSCT treatment and monitored using high-throughput sequencing. By using the ImmunoSEQ, Weng et al. [2013] were able to monitor recurrence of tumor clones that were a precursor of disease relapse using plots of percentage of malignant clone. Spike-in experiments were also performed that showed the assay could detect tumor clones with a sensitivity of 1:150,000.

Cooper et al. [2013] studied how *BRAF* inhibition affects the T-cell repertoire in patients

with metastatic melanoma. Serial biopsies from 8 patients were sequenced prior to treatment and 10-14 days after treatment using the ImmunoSEQ assay. T-cell diversity was calculated pre- and post-treatment and the study found that treatment with *BRAF* inhibitors (BRAFi) resulted in an increase in T-cell diversity in 7 out of 8 patients. The study also found that approximately 80% of the clones found in patients post-treatment were not present before treatment suggesting BRAFi treatment results in an influx of T-cells. Finally, the study found that the proportion of dominant clones present both pre- and post-treatment had an association with patient outcome. Patients with a high-proportion of pre-existing clones post-treatment had a better clinical outcome. It was unclear what specific statistical tests were performed.

Robert et al. [2014] also studied patients with metastatic melanoma but investigated treatment with drugs that inhibit cytotoxic T-lymphocyte-associated protein 4 (CTLA4) instead of BRAFi. CTLA4 inhibition can result in tumor remissions due to a T-cell response but this response has not been fully described. This study had two major conclusions. First, T-cell diversity increases post-treatment but does not seem to be related to the patients' ability to fight cancer. Diversity was calculated using the Shannon diversity index which is defined as

$$H = -\sum_{i=1}^{L} p_i \log(p_i) \tag{7}$$

where p_i is the probabiltiy of CDR3 length i. Second, patients with toxicity had a significantly higher number of unique productive sequences after treatment. Because the authors had a large enough sample the Wilcoxon signed-rank test was used to compare diversity measures pre- and post-treatment. The authors concluded that these results point to a general activation of the immune system rather than a specific anti-cancer immune response.

Muraro et al. [2014] studied the T cell repertoire post-HSCT in 24 multiple sclerosis (MS) patients. HSCT can be used as a way to reset the immune system in MS patients that have autoimmune disease (an organisms immune response to its own cells). The end point

of interest is a non-autoimmune repertoire. Samples were sequenced prior to treatment, two months after treatment and one year after treatment. The study found that patients that had a higher T-cell diversity at two months had a better clinical outcome. In this case diversity was the percentage of T cell repertoire that the top 100 clones included. It was unclear what statistical tests were performed to test for differences.

Finally, Zhu et al. [2013] and Neller et al. [2012] used the ImmunoSEQ assay to examine the T-cell repertoire in patients with herpes virus infection. Both studies showed that virus specific clonotypes are persistent in the patient's T-cell repertoire. This suggests that there is a mechanism to promote a prompt response to viral recurrence.

1.6 Overview of Statistical Methods for CDR3 Data

Spectratype analysis is universally accepted as a procedure to monitor and calculate CDR3 length distributions. However, no agreement has been reached on a method to statistically compare individual repertoires. There are two major hurdles when analyzing CDR3 spectratype data. The first is extracting information from each CDR3 distribution and the second is integrating all of the information from all the CDR3 distributions [Miqueu et al., 2007]. Initially, spectratype analysis was done by "eye-balling" the distribution and placing it into a descriptional category. However, this methodology suffers from variation due to researchers having different ideas about which categories different distributions should fit into. Visual inspection and categorization is also an extremely time consuming method. Hence, researchers have used several computational methods to describe CDR3 distributions. Gorochov et al. [1998] described a method for measuring CDR3 distribution perturbation from a control distribution. This method is applied to T-cell repertoires during progression of AIDS and describes the distributions as a whole. Collette et al. [2003] took this a step further and described an OligoScore which describes each peak individually. This method can be used to identify recurrent peaks in different samples. Venturi et al. [2007] adapted a method from ecology called Simpson's diversity index to measure the diversity in a CDR3

distribution. This method was applied to assess the T-cell repertoire recovery after HSCT by van Heijst et al. [2013]. All these methods assign scores to which statistical methods can then be applied. Kepler et al. [2005] described a statistical method that used the Kullback-Leibler divergence as a measure of how different CDR3 length distributions are from each other. Most recently, Bolkhovskaya et al. [2014] described a non-parametric method for comparing TCR distributions. In the following subsections each method is clearly described.

1.6.1 CDR3 Distribution Perturbation

The CDR3 length distribution measure was applied to measure T-cell repertoires during progression to AIDS. This is a measure of how different TCR distributions are from each other. Gorochov et al. [1998] used data that was translated into a probability distribution from Immunoscope analysis. The area under each peak was measured to calculate a probability using the following formula

$$p_i = \frac{A_i}{\sum_i A_i} \tag{8}$$

where A is the area under each peak (CDR3 length) and $i = 1, \ldots, L$ where L is the number of amino acid lengths in the data. Modifying this notation, let p_{ik}^f represent the probability of peak i of TCRβ family f (VDJ combination) from sample k. The method then calls for a control distribution for each TCRβ family. This was done by taking the average of the control samples denoted by

$$p_{ic}^f = \sum_{k \in c} \frac{p_{ik}^f}{|c|} \tag{9}$$

where c represents the indices of the control samples and $|c|$ is the cardinality of c. The perturbation of each peak from the control for sample k is then defined as

$$D_{ik}^f = p_{ik}^f - p_{ic}^f. \tag{10}$$

The sum of the absolute value of the peak perturbations is then

$$D_k^f = 100 \times \sum_i \frac{|D_{ik}^f|}{2} \tag{11}$$

for sample k and TCRβ family f which yields an overall measure of perturbation for that TCR profile in percentages for each TCRβ family. This is the generalized Hamming distance and measures the distance between two strings of equal size. This measure ranges from 0 to 100 where 0 represents no difference from the control and 100 represents no overlap with the control distribution. A further measure of overall perturbation for a sample k is defined as

$$D_k = \sum_{f=1}^{F} \frac{D_k^f}{F} \tag{12}$$

where F is the number of TCRβ families.

Different statistical tests can then be applied to the measures of perturbation to compare different groups. Gorochov et al. [1998] chose to use the Wilcoxon Rank sum test to compare groups. However, depending on sample size and distributional assumptions, a t-test may be appropriate as well. The the null hypothesis for this test, assuming a Gaussian distribution, would be that the mean D_k is the same across all groups and the alternative hypothesis is that at least one group's mean is different. The hypotheses would be the same when using D_k^f except there would be many tests done, one for each TCRβ family. In this case a multiple comparisons adjustment needs to be applied. For a more detailed discussion of multiple comparisons see section 1.6.8.

1.6.2 OligoScores

Collette et al. [2003] described a score to measure each peak within a group. The OligoScore is defined for the i^{th} peak of the k^{th} sample as

$$OligoScore(i) = \exp(1) \times \sqrt[N]{\prod_{k=1}^{N} p_{ik} \exp(-n_k)} \tag{13}$$

where p_{ik} is probability of the i^{th} peak for the k^{th} sample (as described in section 1.6.1), n_k is the number of peaks in each sample and N the number of samples [Collette and Six, 2002]. This score gives a measure of each peak within a group for each TCRβ family, f. Oligoscores can be interpreted as a score that measures if the distribution has a major peak or not (higher scores mean a peak is major). Collette and Six [2002] did not do any hypothesis testing and instead ranked all oligoscores within each group. The highest oligoscore peaks were then looked at graphically. This score can be adapted to compute peaks for each VDJ combination.

1.6.3 Simpson's Diversity Index

Slow T-cell recovery and low TCR diversity are risk factors for infection, cancer relapse and graft-versus-host-disease (GVHD) after hematopoietic stem cell transplantation (HSCT) [van Heijst et al., 2013]. See section 1.5.1 for a detailed description of GVHD and HSCT. Hence, measuring TCR diversity post-HSCT is an important way to monitor patient health. van Heijst et al. [2013] suggested using the inverse Simpson's diversity index ($\frac{1}{D_S}$). This measure ranges from 1 to ∞ where 1 is no diversity and ∞ is high diversity. The Simpson's diversity index in terms of TCR diversity using unique CDR3 length is the probability that any two TCR's chosen at random from the sample will have different CDR3 lengths and is defined as

$$D_S = 1 - \sum_{i=1}^{L} \frac{n_{ikf}(n_{ikf} - 1)}{n_{kf}(n_{kf} - 1)} \tag{14}$$

where n_{ikf} is the size of the i^{th} TCR CDR3 length (the number of copies of each TCR within a CDR3 length and equivalent to the peak described in section 1.6.1) for the k^{th} sample in TCRβ family, f. L is the number of different TCR CDR3 lengths in the sample and n_{kf} is the total number of TCR sequences sampled in sample k of TCRβ family, f. The diversity index is computed for each TCRβ family. This measure can then be used to compare groups using t-tests or other inferential methods. Further, it can be adjusted to be computed for

each VDJ combination.

van Heijst et al. [2013] were not interested in comparing groups but time points. Using paired t-tests they found the patient TCR diversity did not change over time. However, using correlations they did find that the composition of the TCR distribution changed drastically. For example, the dominant peak from one time point to the next could be completely different depending on what antigens the patient was in contact with recently.

1.6.4 Kullback-Leibler Divergence Method

Kepler et al. [2005] described this method directly for spectratype data which results in relative abundance measures as described in section 1.2. Let's consider the multinomial distribution in terms of CDR3 length distribution. In this case, L is the number of unique CDR3 lengths, $\mathbf{q}$ is a vector of probabilities of each CDR3 length occuring, m_i is the number of counts of the i^{th} CDR3 length and n_k is the total number of counts within a sample. Thus, the probability mass function (pmf) of the relative abundance of the lengths of CDR3 clones, $\mathbf{p} = \frac{\mathbf{m}}{n}$ is

$$f_p(\mathbf{p}|n_k, \mathbf{q}) = n_k^{L-1} C_{n_k}(n_k\mathbf{p}) \prod_{i=1}^{L} q_i^{p_i n_k}, \tag{15}$$

where $C_{n_k}(n_k\mathbf{p})$ is the multinomial coefficient and $\mathbf{q}$ is a vector of probabilities corresponding to each category i.

$$C_{n_k}(n_k\mathbf{p}) = \frac{\Gamma(n_k + 1)}{\prod_{i=1}^{L} \Gamma(n_k p_i + 1)} \tag{16}$$

for $\sum_{i=1}^{L} n_k p_i = n$ and zero otherwise.

The Stirling approximation can then be applied to the multinomial coefficient to simplify its form assuming that for each i, $n_k p_i$ is large enough.

$$\log C_{n_k}(n_k\mathbf{p}) = \log\Gamma(n_k+1) - \sum_{i=1}^{L}\log\Gamma(p_i n_k+1) \tag{17}$$

$$= \sum_{i=1}^{L}\left[-n_k p_i \log p_i - \frac{1}{2}\log p_i\right] - \frac{L-1}{2}\log 2\pi n_k + O(\frac{1}{n_k}) \tag{18}$$

Plugging this back into the pmf for $\mathbf{p}$ we get

$$f_r(\mathbf{p}|n_k,\mathbf{q}) = \frac{n_k^{(L-1)/2}e^{-n_k D(\mathbf{p};\mathbf{q})}}{\sqrt{(2\pi)^{L-1}\Pi_{i=1}^{L}p_i}}\delta(0) \tag{19}$$

where δ is the Dirac delta, $p. = \Sigma_i p_i$ and $D(\mathbf{p};\mathbf{q})$ is the Kullback-Leibler divergence.

$$D(\mathbf{p};\mathbf{q}) = \Sigma_{i=1}^{L} p_i \log\frac{p_i}{q_i} \tag{20}$$

where $p. = q. = 1$.

To determine the distribution of the Kullback-Leibler divergence we can apply a cumulative generating function method where

$$h(s) = \log\int d^L p \mathrm{e}^{sD(\mathbf{p};\mathbf{q})} f_r(\mathbf{p}|n_k,\mathbf{q}) \tag{21}$$

$$= -\lambda\log(1-\frac{s}{n_k}) \tag{22}$$

This is the cumulative generating function of a gamma random variable with $\lambda = (L-1)/2$ and scale $= \frac{1}{n_k}$. The the distribution of the Kullback-Leibler divergence is given by

$$f_D(D|n_k) = \frac{n_k^{\lambda}}{\Gamma(\lambda)}D^{\lambda-1}\mathrm{e}^{-n_k D} \tag{23}$$

Using the above derivations we can compare group distributions of the CDR3 length. We will be testing to see if the population parameter $\mathbf{q}$ is identical in all groups. Thus, our null

hypothesis is that the distribution of CDR3 lengths is identical in all groups.

Let p_{ijk} represent the relative frequency counts of CDR3 length $i = 1, ..., L$, where $j = 1, ..., G$ represents the group and $k = 1, ..., n_{ij}$ the k^{th} subject of the j^{th} group for CDR3 length i. Then the mean of group j for CDR3 length i is defined as

$$\bar{p}_{ij.} = \frac{1}{n_i} \sum_{k=1}^{n_j} p_{ijk} \tag{24}$$

where n_i is the number of counts of CDR3 length i and n_j is the number of subjects in group j. If G is the number of groups then the grand sample mean is

$$\bar{p}_{i..} = \frac{1}{n_.} \sum_{j=1}^{G} n_i p_{ij.} \tag{25}$$

where $n.$ is the total number of subjects.

If we denote $\mathbf{p}_{jk}$ as the vector that has components p_{ijk} we can partition the total divergence as

$$D_{tot} = \sum_j \sum_k D(\mathbf{p}_{jk}; \mathbf{q}) \tag{26}$$

$$= \sum_j \sum_k [D(\mathbf{p}_{jk}; \bar{\mathbf{p}}_{k.}) + D(\bar{\mathbf{p}}_{k.}; \bar{\mathbf{p}}_{..}) + D(\bar{\mathbf{p}}_{..}; \mathbf{q})] \tag{27}$$

Under the null hypothesis that $\mathbf{q}$ is the same in all groups the expected values of the partitioned divergences are

$$E[D(\mathbf{p}_{jk}; \bar{\mathbf{p}}_{k.})] = \frac{L-1}{n_k}(1 - \frac{G-1}{n.}) \tag{28}$$

$$E[D(\bar{\mathbf{p}}_{k.}; \bar{\mathbf{p}}_{..})] = \frac{L-1}{n_k n.}(1 - \frac{1}{G}) \tag{29}$$

$$E[D(\bar{\mathbf{p}}_{..}; \mathbf{q})] = \frac{L-1}{n.n_k} \tag{30}$$

Finally, because the divergences are distributed Gamma, the following statistic is asymptotically distributed as Fisher's F with (L-1)(G-1) numerator and (L-1)(n.-G+1) denominator degrees of freedom under the null hypothesis.

$$F = \frac{(n. - G + 1)\Sigma_k D(\bar{\mathbf{p}}_{k.}; \bar{\mathbf{p}}_{..})}{(G-1)\Sigma_{jk} D(\mathbf{p}_{jk}; \bar{\mathbf{p}}_{k.})} \tag{31}$$

Notice that the numerator is a between group divergence and the denominator is within group divergence. This is analogous to the typical ANOVA test. This statistic can be used to compare different groups of subjects that have TCR data collected for them at each VDJ combination, f.

1.6.5 Non-parametric Method

Bolkhovskaya et al. [2014] described a non-parametric method for assessing the TCR length distribution. To incorporate statistical uncertainty, a non-parametric kernel distribution estimator is constructed for the cumulative frequency distribution, $F_f(p^*) \equiv \text{Prob}(p \geq p^*)$

$$\hat{F}_f(p) = \frac{1}{L} \sum_{i=1}^{L} \Phi(p - p_i), \tag{32}$$

where L is the number of TCR lengths, p_i is the frequency of each TCR CDR3 length, p is a pre-chosen frequency value and $\Phi(\cdot)$ is the cumulative normal distribution with mean zero and standard deviation

$$\sigma_{p_i} = \left[\sum_{s=1}^{S} \sigma_{p_i,s}^2 \right]^{\frac{1}{2}} \tag{33}$$

where $\sigma_{p_i,s}^2$ is the standard deviation present at each of S steps of repertoire profiling. Three steps (S) during the sampling process that add to statistical error are sampling T-cells, PCR and sequencing. Each of these steps introduce variation to the final TCR frequency. Bolkhovskaya et al. [2014] assume the sampling process at each step takes on a binomial distribution so

$$\sigma^2_{p_i,s} = \sqrt{\frac{p_i(1-p_i)}{N_s}} \tag{34}$$

where N_s is the total number of samples at step s. However, this variation can only be measured at the sequencing stage so $S = 1$. From here the probability density distribution can be estimated by

$$\hat{W}(p) = -\frac{d}{dp} = \hat{F}_f(p). \tag{35}$$

Finally, the upper 95% confidence interval can be computed using

$$p_0 = 1 - (1 - 0.95)^z, z = \sum_{s=1}^{S} \frac{1}{N_s}. \tag{36}$$

Bolkhovskaya et al. [2014] applied this method to patient data and found that $F_f(p)$ follows a power law decay

$$F_f(p) \propto p^{\alpha_i}. \tag{37}$$

The power law represents a relationship between two variables where one variable varies as the power of another. It can be seen in scenarios where a few values dominate. Many times a TCR CDR3 length distribution is dominated by one or two lengths. Thus, the power may be an appropriate way to compare TCR CDR3 length distributions between groups. This can be done by calculating and comparing α values between groups.

1.6.6 Proportion Logit Transformation

The proportion of shared sequences between donor and recipient can be considered as a measure of how closely the donor and recipient are matched. The question posed is if there is a difference in the proportion of shared sequences between donor and recipient between patients with GVHD and no GVHD. Hence, we can match recipient and donor

TCR sequencing data by amino acid sequence and calculate the proportion of unique and shared TCR sequences. Proportions do not follow a Gaussian distribution. However, by applying a logit transformation to the proportions, they can be transformed into a value that follows a Gaussian distribution. If we let π be the proportion of shared sequences, the logit transformation is defined as:

$$\text{logit}(\pi_{kf}) = \log(\frac{\pi_{kf}}{1-\pi_{kf}}) \tag{38}$$

where π_{kf} is the proportion of shared sequences for subject k within VDJ family, f. Once π_{kf} is logit transformed, t-tests can be applied to test for group mean differences. Since each VDJ combination is to be tested a multiple comparisons correction is needed. A discussion of multiple comparisons can be found in section 1.6.8.

1.6.7 Tabular Summary of Statistical Methods for CDR3 data

Table 1 shows a summary of the methods described and test that is being performed.

Table 1: Table of Methods for Analyzing TCR Sequencing Data

Method	Summaries	Alternative Hypothesis
Perturbation using Matched Donor	Each VDJ combination	Group pertrubations are different
Perturbation using Mean Distribution	Each VDJ combination	Group pertrubations are different
Oligoscores	Each CDR3 length within a VDJ combination	N/A
Simpson's Diversity Index	Each VDJ combination	Group diversity measures are different
Kullback-Leibler Divergence	Each VDJ combination	Group distributions are different
Non-parametric Method	Each VDJ combination	Group α values are different
Proportion Logit Transformation	Each VDJ combination	Group proportions are different

1.6.8 Multiple Comparisons

Testing many independent hypotheses runs the danger of identifying significant differences that are the result of random chance. Methods such as the Bonferroni correction aim to control the probability of making a Type I error. This means that these methods also control the simultaneous correctness of all rejections [Benjamini and Hochberg, 2000]. However, the Bonferroni method is conservative and results in a substantial loss in power for individual tests. For high-throughput technologies it is more important to find all possible differences at the cost of getting a few hypotheses wrong.

The false discovery rate is defined as the expected ratio of erroneous rejections to the number of rejected hypotheses. Benjamini and Hochberg [1995] showed that this procedure has a substantial increase in power over classic α controlling methods. The procedure was shown to control the FDR at a lower level than desired so the adaptive procedure was created [Benjamini and Hochberg, 2000].

Let m represent the total hypotheses that we wish to test and m_0 represent those that are true. Let R define the total number of hypotheses rejected and V denote the number of those hypotheses that were rejected incorrectly. Note that V is an unobservable variable. Using these definitions the effective error rate is $E[\frac{V}{m_0}]$ and the type I error rate is $P(V \geq 1)$. By testing each hypothesis at level α we guarantee $E[\frac{V}{m_0}] \leq \alpha$ but not $P(V \geq 1) \leq \alpha$. Using a procedure such as the Bonferroni by testing each hypothesis at $\frac{\alpha}{m}$ guarantees $P(V \geq 1) \leq \alpha$. However, in the FDR procedure the error of falsely rejecting a null hypothesis is captured by the random variable $Q = \frac{V}{R}$, the proportion of false-positives [Benjamini and Hochberg, 1995]. Thus,

$$FDR = E(Q) = E\left(\frac{V}{R}\right).$$

Benjamini and Hochberg [2000] showed that when $m_0 < m$ the FDR is smaller than or equal to the Type I error rate. Thus any procedure that controls the Type I error rate also

controls the FDR. But if you are only controlling the FDR then an improvement in power is expected. The following procedure is shown to control the FDR at level q and improve power:

1. Let $P_{(1)} \leq ... \leq P_{(i)} \leq ... \leq P_{(m)}$ be the ordered p-values.
2. Let k be the largest i for which $P_{(i)} \leq \frac{i}{m}q$.
3. Reject all k ordered hypotheses.

The proof that this procedure controls the FDR is based on the lemma that says for $m_1 = m - m_0$ false null hypotheses the test procedure satisfies the inequality [Benjamini and Hochberg, 2000]

$$E(Q|P_{m_0+1} = p_1, ..., p_m = p_{m_1}) \leq \frac{m_0}{m}q$$

Thus, when some of the null hypotheses are rejected the FDR is controlled at a level less than q. So there is once again room to improve power in this procedure. If m_0 was known then $E(V) = m_0\alpha$ and $r(\alpha) = v + s$ where s is the number of null hypotheses rejected correctly. So the new estimate of the FDR can be [Benjamini and Hochberg, 2000]

$$\hat{Q}_e(\alpha) = \frac{\hat{v}(\alpha)}{r(\alpha)} = \frac{\hat{m}_0\alpha}{r(\alpha)}.$$

Then to control the FDR at level q we can follow the following procedure

1. Let k be the largest i for which $P_{(i)} \leq \frac{i}{\hat{m}_0}q$.
2. Reject all k ordered hypotheses.

The final problem is how to estimate m_0. A graphical method can be used. If you consider the p-values to be independent, under the null hypothesis they should follow a uniform[0,1] distribution. Thus, a quantile plot of the p_i's should have a linear relationship through the

origin and slope of $S = \frac{1}{m+1}$. When there are false null hypotheses they tend to stack at the lower end. However, the upper end should continue with a linear relationship. Thus, we can use the slope of the higher p-values to solve for m_0. Benjamini and Hochberg [2000] suggest the estimate of m_0 to be calculated as follows.

1. Calculate slopes $S_i = \frac{1-p_i}{m+1-i}$ and continue as long as $S_i \geq S_{i-1}$
2. Stop once $S_j < S_{j-1}$.
3. Then $\hat{m}_0 = \min(\frac{1}{S_j} + 1, m)$.

1.7 Discussion

In this chapter we discussed T-cell biology and the ImmunoSEQ platform used to analyze the T-cell repertoire. Further, different methodologies were described to analyze the high-throughput sequencing data generated by the ImmunoSEQ assay. In the next chapter, we will apply these methodologies to a hematopoietic stem cell transplantation data set. In Chapter 3, we will discuss how high-throughput sequencing data can be considered compositional data and the analytical challenges that can present. Finally, the same HSCT data from chapter 2 will be analyzed as compositional data.

2 Applications to TCR sequencing data

2.1 Introduction

In this chapter, HSCT data (described in section 2.3) were analyzed using the methodologies described in chapter 1. To determine what pre-processing steps other researchers used, previously published data were reanalyzed. Finally, the differences in the methods were discussed.

2.2 Reanalysis of Published TCR data

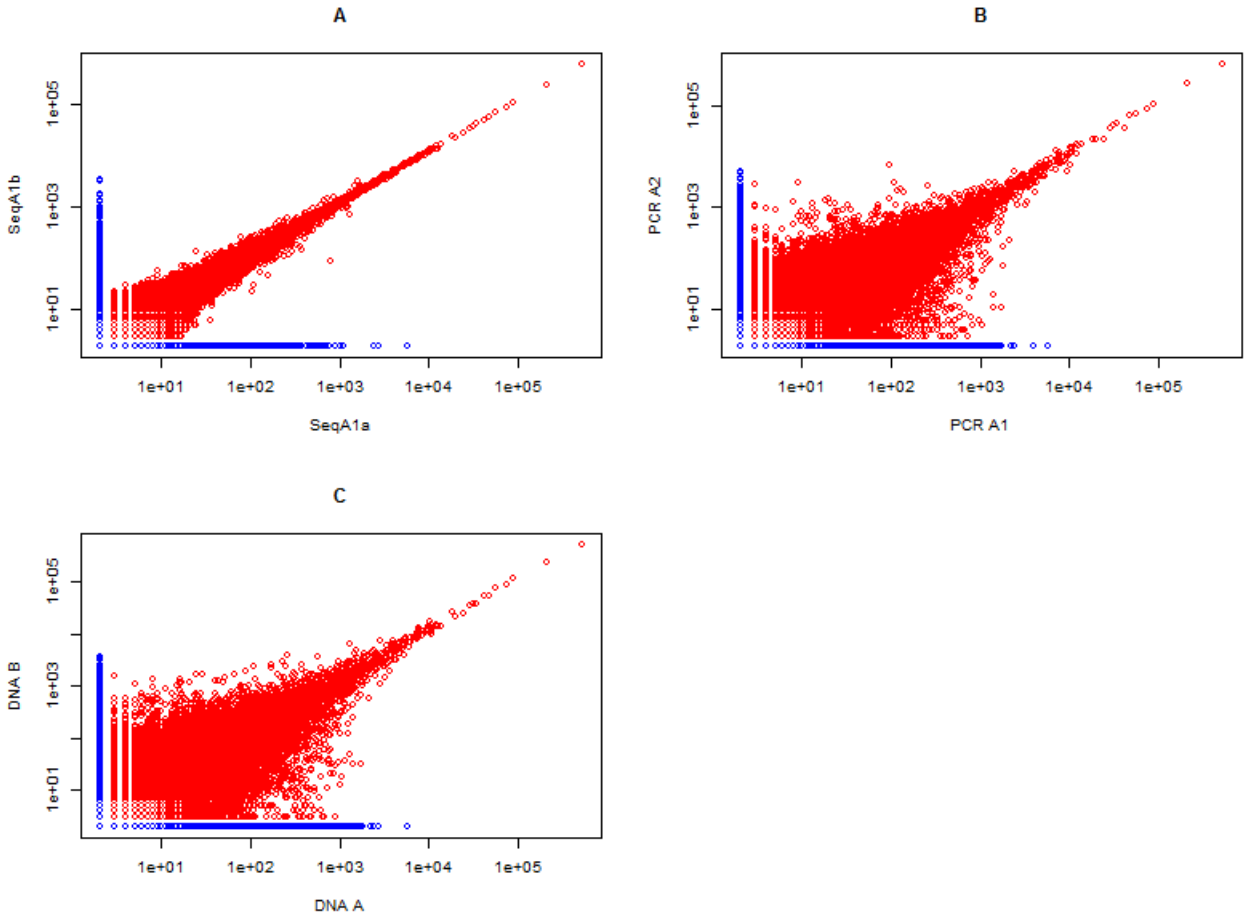
We acquired the data from Dr. Harlan Robins' lab that were analyzed in the 2012 paper titled "Ultra-sensitive detection of rare T-cell clones" [Robins et al., 2012]. This article describes the Adaptive Technologies ImmunoSEQ technology and it's ability to detect T-cell clones. Thus, it was useful to reanalyze the data to determine the exact process used to perform the analysis. Specifically, we acquired replicate data for one patient but were unable to obtain the spike-in data used in the experiments. The data that we acquired included samples DNA A, DNA B, PCR A1, PCR A2, SeqA1b and SeqA1a.

We performed a similar analysis and compared our results to that reported by Robins et al. [2012]. In the publication, the authors were not specific about their data processing procedures so several different attempts were made to replicate their results. The first step required deciding whether to use all the sequences or a subset. The sequences are flagged by Adaptive Biotechnologies software as productive (usable proteins) or non-productive and through trial and error we discovered both productive and non-productive sequences were used for the analysis performed by Robins et al. [2012]. Next, in order to compare counts between replicates the files needed to be merged. The CDR3 sequences can be merged either by amino acid or nucleotide sequence and through trial and error we discovered Robins et al. [2012] merged by nucleotide sequence.

Comparing Figure 6 to Figure 2 reported in the Robins et al. [2012] paper, we see that the

log-log plots are almost identical. Thus we established in our reanalysis the specific process the authors undertook. We noted a small difference between the total number of shared sequences between samples SeqA1a and SeqA1b. The authors identified 150,612 sequences but our analysis identified 150,567.

Figure 6: **Reproducibility Plots** The plots show frequencies of unique sequences from different samples.



2.3 Data

Thirteen patients with hematological disorders who underwent HSCT were included in this study. This was a randomized phase II trial conducted at Virginia Commonwealth University

of a reduced intensity condition regimen for patients undergoing HSCT [Meier et al., 2013]. Human leukocyte antigen (HLA) matching was done and only patients with 7 of 8 or 8 of 8 matches were eligible for transplantation. HLA is a protein found on most cells in the body and there are 8 major types. The best transplant outcomes happen when patient and donor HLA's are closely matched. Note that the TCR's present are also determined by the protein they recognize along with the HLA's present. Thus, the sample data could be skewed despite the close HLA matching. Peripheral blood stem cells were collected from the donor and recipient whole blood samples were collected post-HSCT. The goal of this analysis was to identify differences at the molecular level between T-cell repertoires of patients who suffered from GVHD and those that did not. Eight patients suffered from GVHD and five did not. T-cell sequencing data was generated using the ImmunoSEQ technology described in section 1.2. The methods described in section 1.6 were applied to the data and the results are described in the following sections.

All data analyses were performed using the R programming environment version 3.1.1 [R Core Team, 2014]. Only sequences that could result in usable proteins (productive sequences) were used in the analyses. Further, reads per million (RPM) were calculated for each sample and used in the analyses. RPM were calculated by dividing each CDR3 frequency by the total number of CDR3 sequences and multiplying by one million. VDJ combinations were filtered out to keep only combinations that had counts for at least 7 patients, leaving 947 VDJ combinations for analysis. CDR3 length summaries were obtained by summing all the CDR3 RPM's of the same length within each VDJ combination. Further data management applied to a specific method will be described in the following sections.

2.4 Permutation Tests

Because of the low sample size (13 patients) permutation tests were used to compare groups. In this case, there are two samples that have been drawn from two distributions that may or many not be the same. That is the no GVHD group is drawn from a distribution, F,

independent of the GVHD group from a distribution, G. Thus, the null hypothesis to test is that the two distributions are equal and the alternative is that they are not equal. That is

$$H_0 : F = G$$

$$H_a : F \neq G$$

Under the null hypothesis, any of the 13 observations could have come from either distribution F or G. All 13 observations were considered as a single set of values. A sample of size five was drawn without replacement from this set of values to represent the no GVHD group. The remaining eight observations represent the GVHD group. 1000 random samples were drawn in this manner. The absolute difference between group means, $|\hat{\mu}^*|$, was calculated for each permutation. The p-value is then calculated as follows

$$\text{p-value} = \frac{\#(|\hat{\mu}^*| \geq |\hat{\mu}|)}{1000} \tag{39}$$

where $|\hat{\mu}|$ is the absolute difference in means using the original sample. Thus, this is the probability that the statistic was greater than that which was observed using the original sample [Efron and Tibshirani, 1994]. P-values were calculated for all 947 VDJ combinations considered for analysis. The p-values were then corrected for multiple comparisons using an FDR threshold of 0.05. All permutation tests and FDR corrections were implemented in the R programming environment.

2.5 CDR3 Distribution Perturbation Results

The CDR3 Distribution Perturbation method described in section 1.6.1 was adapted for TCR sequencing data and applied to the study data. Each possible VDJ combination, f, was examined individually. There were 947 perturbations calculated for each sample after filtering. The perturbations were calculated both as a perturbation from each donor's T-cell

distribution, D^f_{kd}, and as a perturbation from an average donor T-cell distribution, D^f_{ka}. D^f_{kd} measures the perturbation from each donor and can be considered as a measure of how similar the recipient is to the donor. D^f_{ka} measures the perturbation from a control distribution and can be considered to measure how different the samples are from each other. Depending on which calculation is used the interpretation of the difference between the GVHD and no GVHD groups is slightly different. Permutation tests described in section 2.4 were used to test for differences between the recipient GVHD and no GVHD groups.

The probability of each CDR3 length, i, was calculated using equation 8. The RPM were summed for each unique CDR3 length and then divided by the total RPM present within the VDJ combination of interest to calculate the CDR3 length probability, A_i. First, the perturbation for each recipient was calculated as a perturbation from their matched donor. The donor CDR3 length probabilities were calculated in the manner described above. The perturbations were also calculated as a perturbation from a mean distribution of the 13 donors. The mean distribution was calculated by averaging the probability of each CDR3 length across all 13 donors. The perturbations were calculated using equation 10, where k is each sample and c is the control distribution. The control distribution is either an average donor distribution or the matched donor distribution.

Then statistics were calculated using each matched donor for each VDJ combination and tested for differences between the GVHD and no GVHD groups. After adjusting for multiple comparisons using the Benjamini and Hochberg FDR method described in section 1.6.8, there were two significant differences (FDR $<$0.001). The distribution of the statistic for the two signifcantly different VDJ combinations can be seen in Figure 7. The figure shows boxplots where the median, 25th and 75th percentiles make up the box. The whiskers extend to 1.5 times the interquartile range (IQR) and if there are outliers they are represented by dots outside the whiskers. Table 2 shows the median perturbations and observed FDR for the two VDJ combinations. It was of interest to determine why the differences are in opposite directions. Looking at Figures 9 and 10 it appears as though the VDJ combination TRBJ2-

6 TRBV9 TRBD1-2 is not present in a large number of the samples. Figure 9 shows the CDR3 length distributions in the donors for VDJ combination TRBJ2-6 TRBV9 TRBD1-2 and Figure 10 shows the CDR3 length distributions in the recipients for VDJ combination TRBJ2-6 TRBV9 TRBD1-2. Matched donors and recipients are in the same positions in the 2 figures. The low prevalence of this VDJ combination in the sample population could be causing the results of the test to be skewed.

Second the statistics were calculated using a mean donor distribution as a control for each VDJ combination. After adjusting for multiple comparisons using the Benjamini and Hochberg FDR method, there were three significant differences (FDR <0.001). The distribution of the statistic for the three significantly different VDJ combinations can be seen in Figure 8. This is a measure of how different the No GVHD and GVHD groups are from each other. Table 3 shows the median perturbations and observed FDR for the three VDJ combinations. Note that the two analyses have no VDJ combinations in common.

Figure 7: Distribution of perturbation from matched donor statistic for the 2 significantly different VDJ combinations.

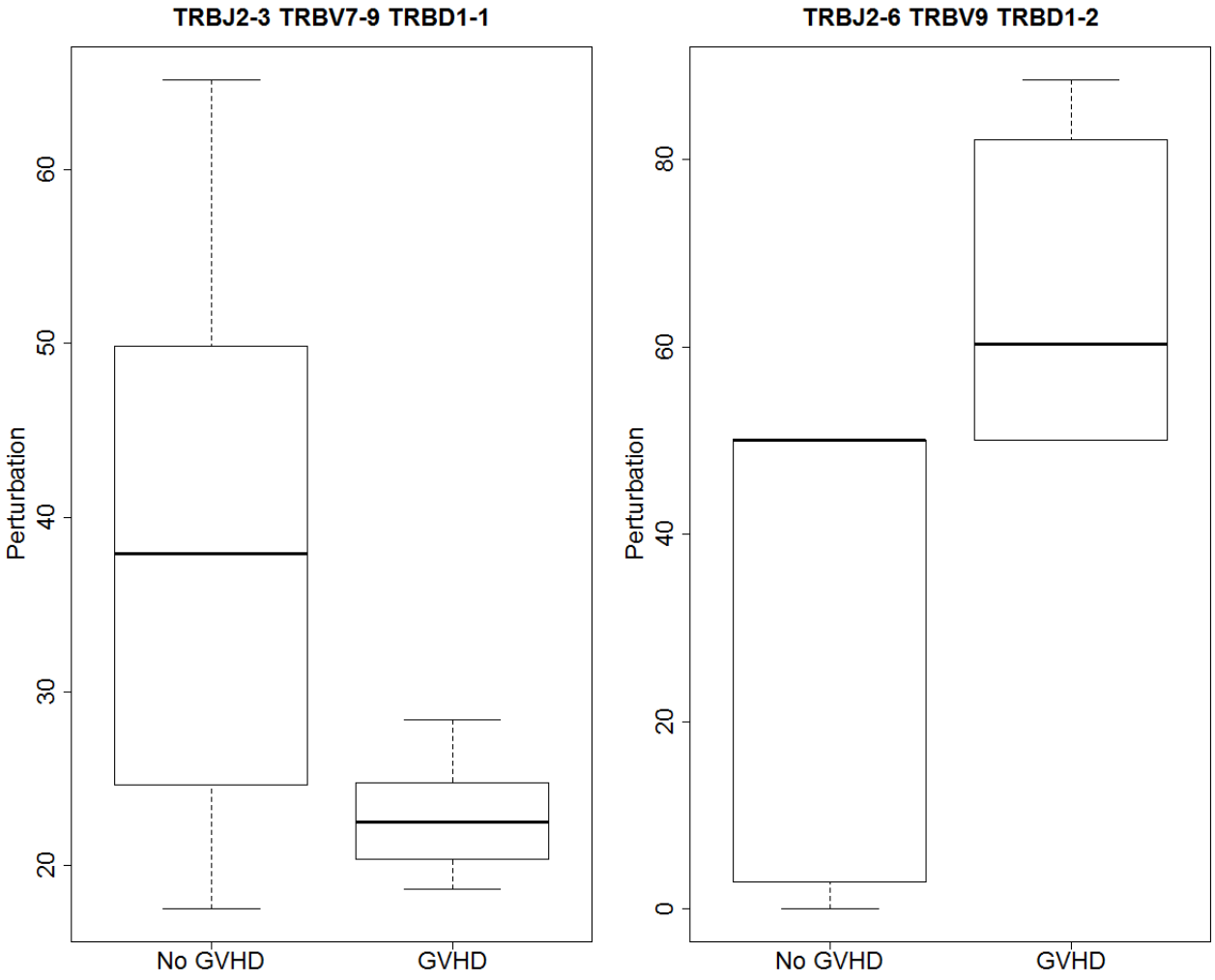


Figure 8: Distribution of perturbation from mean donor statistic for the 3 significantly different VDJ combinations.

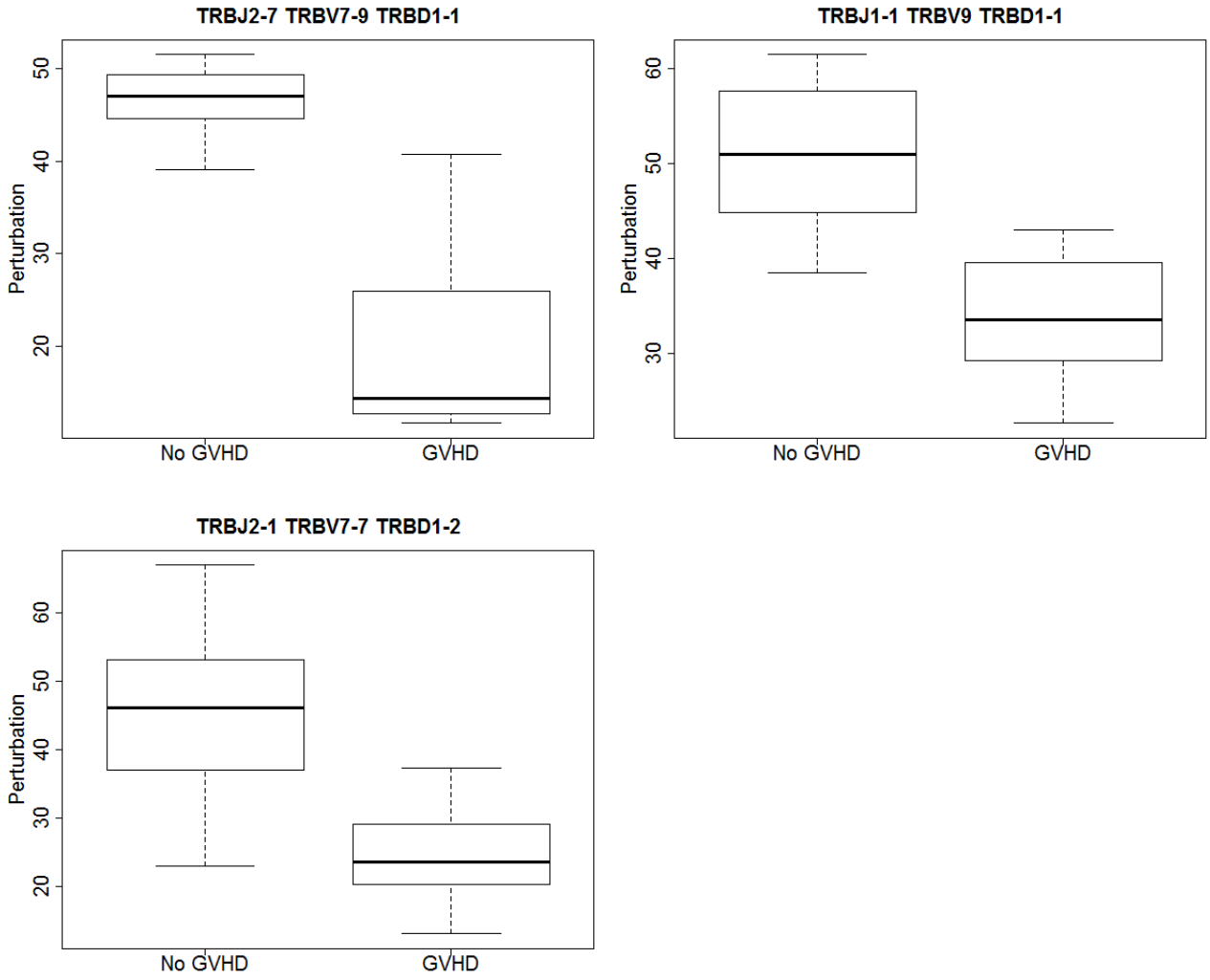


Table 2: Median Perturbation Using Matched Donor Control

JGene	VGene	DGene	Median No GVHD Perturbation	Median GVHD Perturbation	Observed FDR< 0.05
TRBJ2-3	TRBV7-9	TRBD1-1	37.946	22.513	<0.001
TRBJ2-6	TRBV9	TRBD1-2	50.000	60.310	<0.001

Table 3: Median Perturbation Using Mean Control

JGene	VGene	DGene	Median No GVHD Perturbation	Median GVHD Perturbation	Observed FDR< 0.05
TRBJ2-7	TRBV7-9	TRBD1-1	47.001	14.364	<0.001
TRBJ1-1	TRBV9	TRBD1-1	51.051	33.529	<0.001
TRBJ2-1	TRBV7-7	TRBD1-2	46.154	23.571	<0.001

Figure 9: Distributions of CDR3 lengths from VDJ combination TRBJ2-6 TRBV9 TRBD1-2 donors.

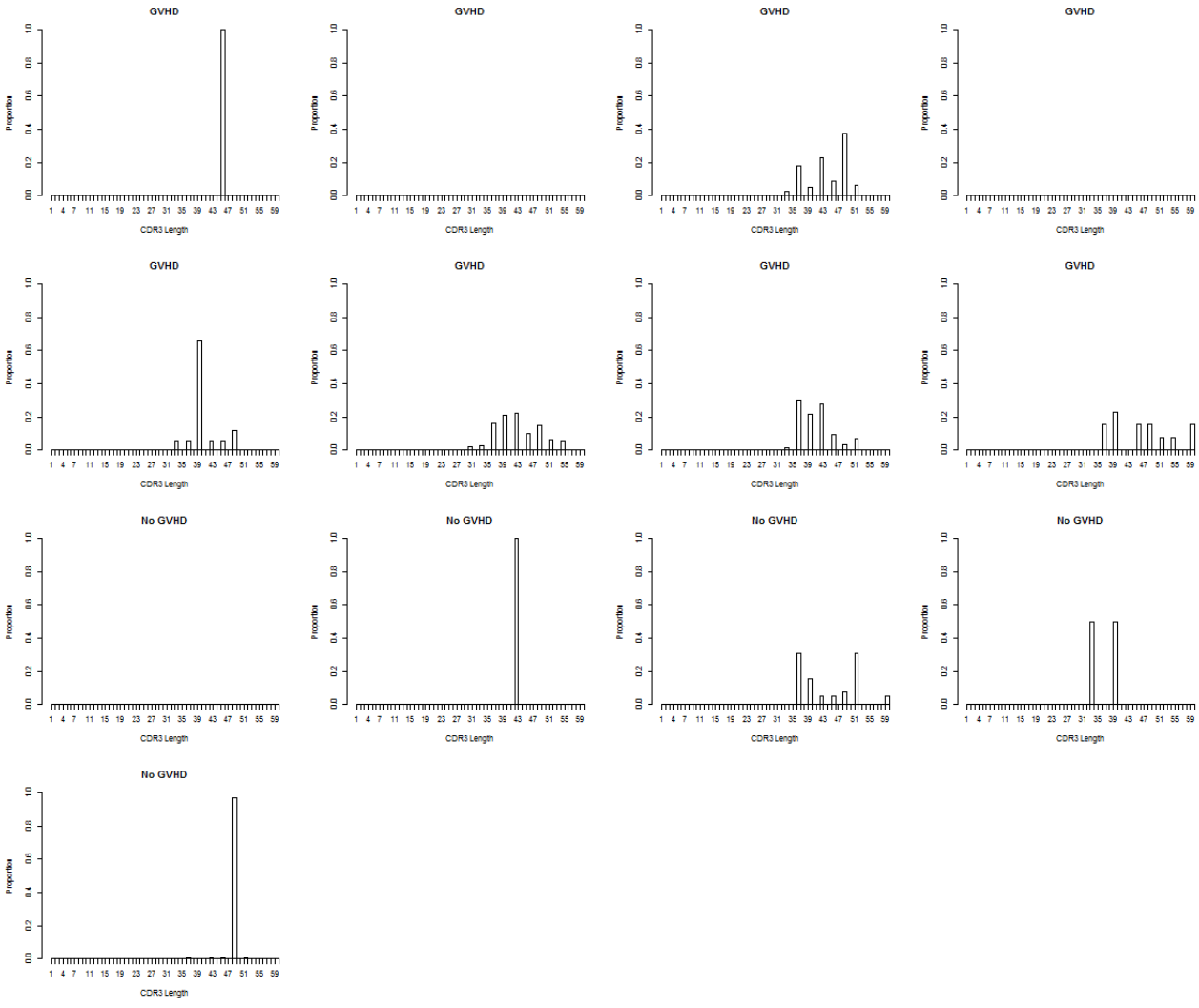
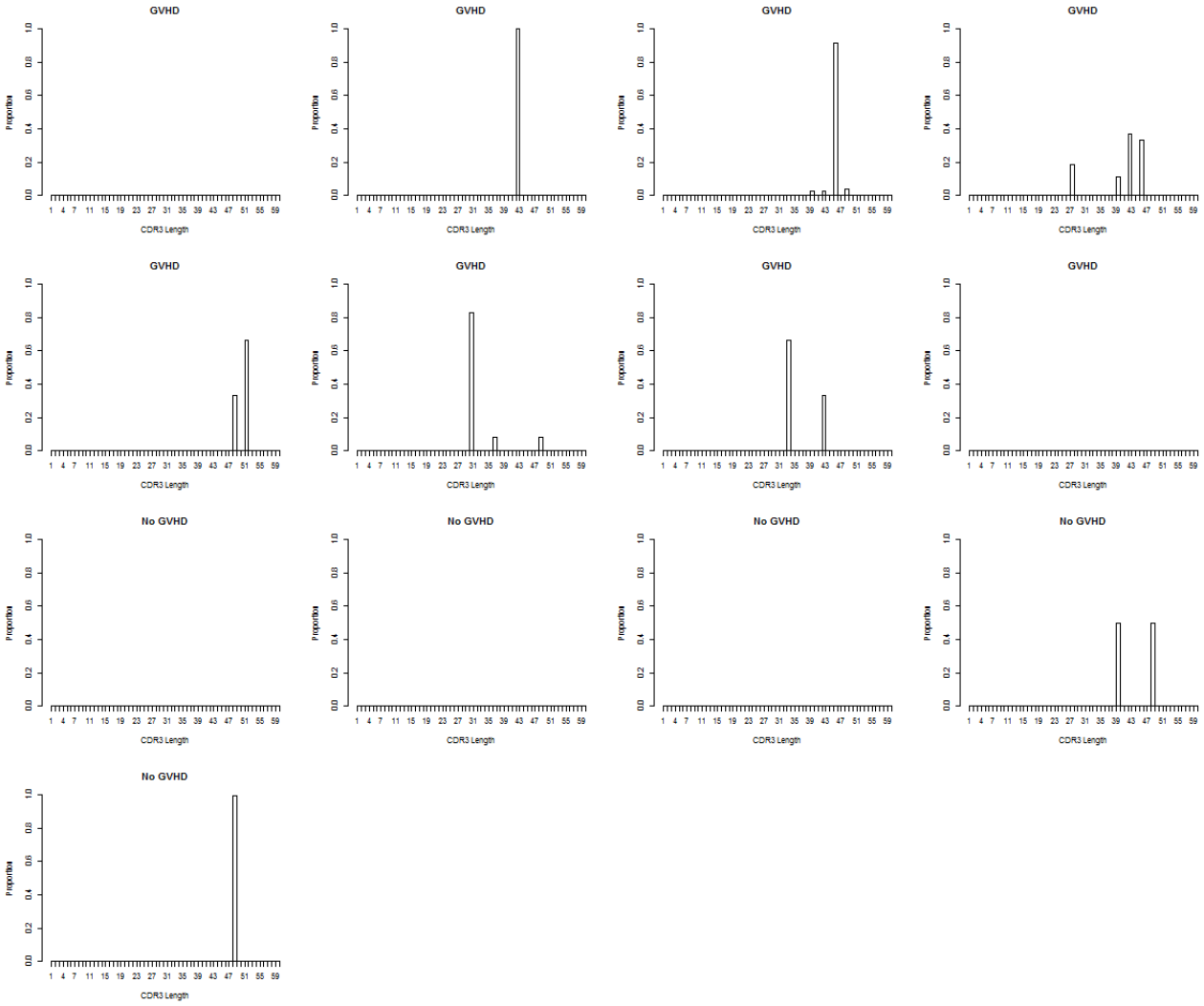


Figure 10: Distributions of CDR3 lengths from VDJ combination TRBJ2-6 TRBV9 TRBD1-2 recipients.



2.6 Oligoscores Results

The Oligoscore method described in section 1.6.2 was adapted for TCR sequencing data and applied to the study data. Each possible VDJ combination, f, was examined individually. An Oligoscore was calculated for each CDR3 length, i, within each VDJ combination using equation 13. The RPM were summed for each unique CDR3 length and then divided by the total RPM present within the VDJ combination of interest to calculate the CDR3 length probability. Further, the scores were calculated for both groups, GVHD and no GVHD, individually. Each CDR3 length, i, was considered that ranged from 1 to n_k, the number of peaks for sample k. Because this is a measure to rank interesting peaks, the maximum Oligoscore from each group was examined graphically. Looking at Figure 11 we see that CDR3 length 39 shows up as a major peak in every patient with GVHD but only 2 of the No GVHD patients. Similarly, looking at Figure 12 we see that CDR3 length 33 shows up in 4 out of 5 patients with no GVHD and 3 out of 8 with GVHD. Further, Fisher's exact test was calculated to test for a difference in number of subjects that had the two peaks as major peaks. Calculating the Fisher's exact test of the peak with the highest oligoscore in the GVHD group showed a significant difference between the two groups (p=0.035). Calculating the Fisher's exact test of the peak with the highest Oligoscore in the no GVHD group showed a non-significant difference between the two groups (p=0.266).

Figure 11: Distribution of CDR3 length for highest Oligoscore in GVHD group.

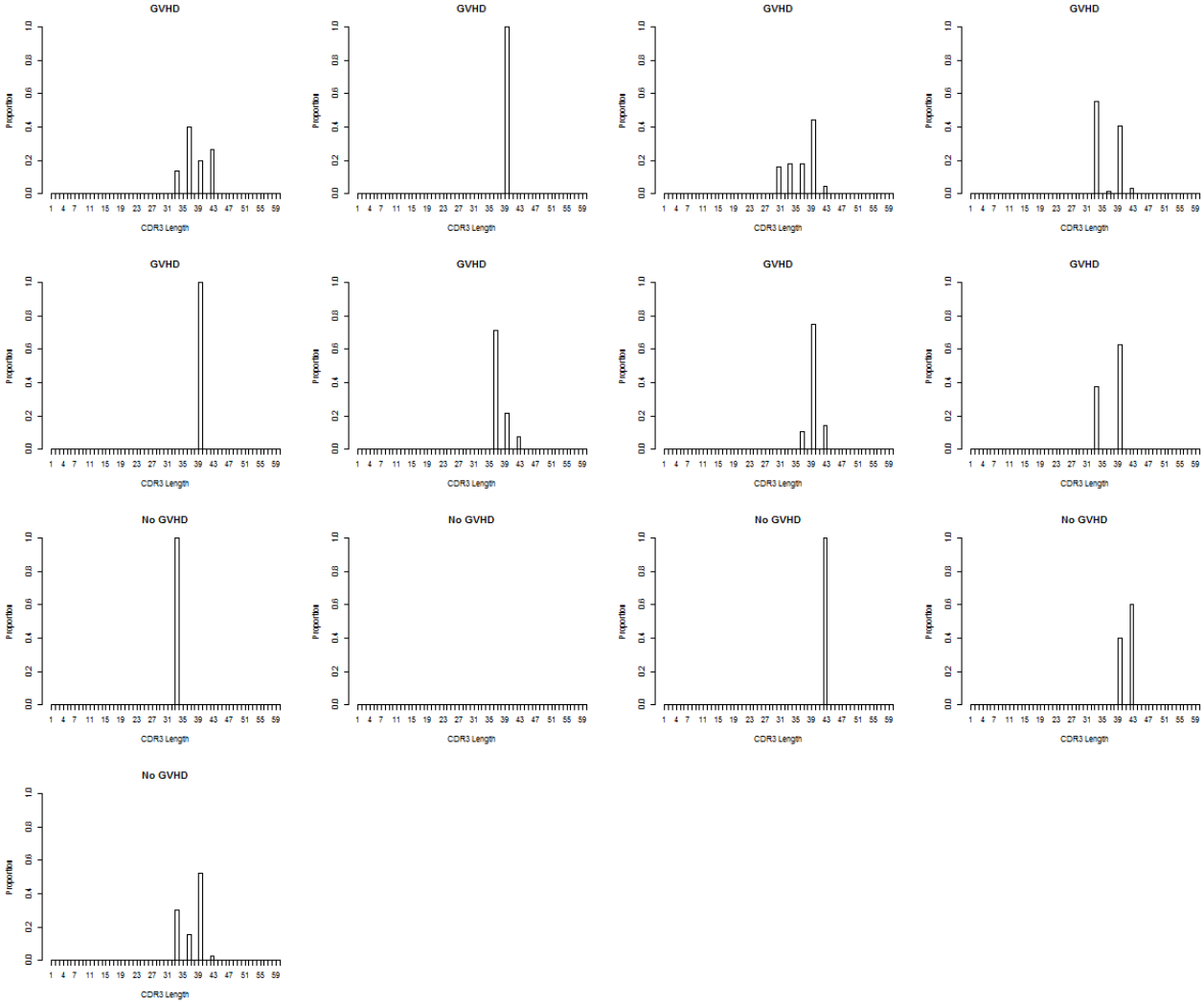
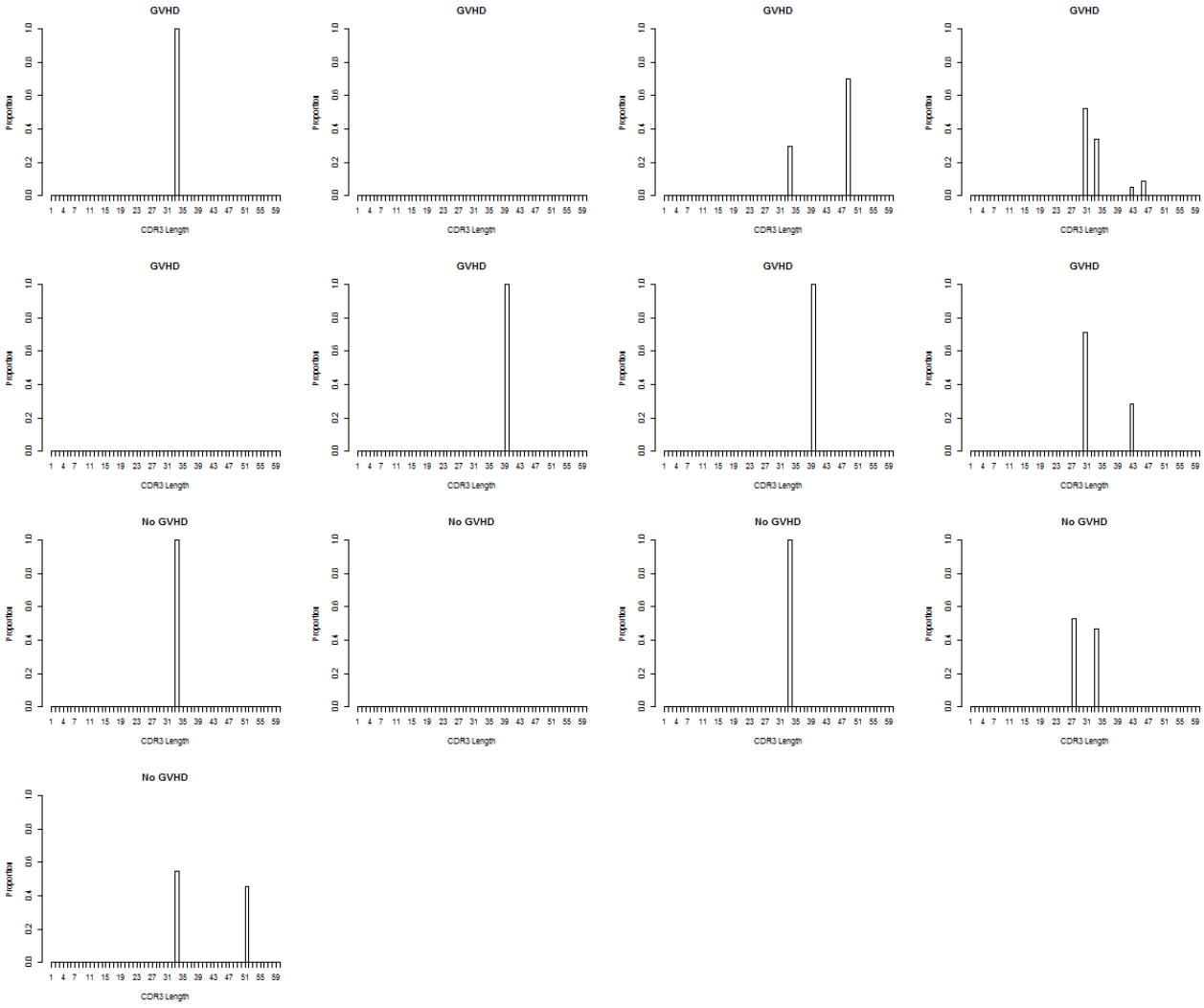


Figure 12: Distribution of CDR3 length for highest Oligoscore in No GVHD group.



2.7 Simpson's Diversity Index Results

The Simpson's diversity index method described in section 1.6.3 was adapted for TCR sequencing data and applied to the study data. Each possible VDJ combination, f, was examined individually. There were 947 diversity indexes calculated for each sample using equation 14. Each CDR3 length, i, was considered that ranged from 1 to L. L could vary for each VDJ combination, n_{kf} was the total RPM for each sample within a VDJ combination and n_{ikf} was the RPM for the i^{th} CDR3 length in the k^{th} sample of VDJ combination,

f. Permutation tests described in section 2.4 were used to test for differences in Diversity between the GVHD and no GVHD groups.

Simpson's diversity indices were calculated for every VDJ combination in each sample. After adjusting for multiple comparisons using the Benjamini and Hochberg FDR method, there were five significant differences (FDR < 0.001). The distribution of the statistic for the five significanlty different VDJ combinations can be seen in Figure 13. Table 4 shows the median diversity and observed FDR for the five VDJ combinations.

Figure 13: Distribution of Simpson's Diversity Index statistic for the 5 significantly different VDJ combinations.

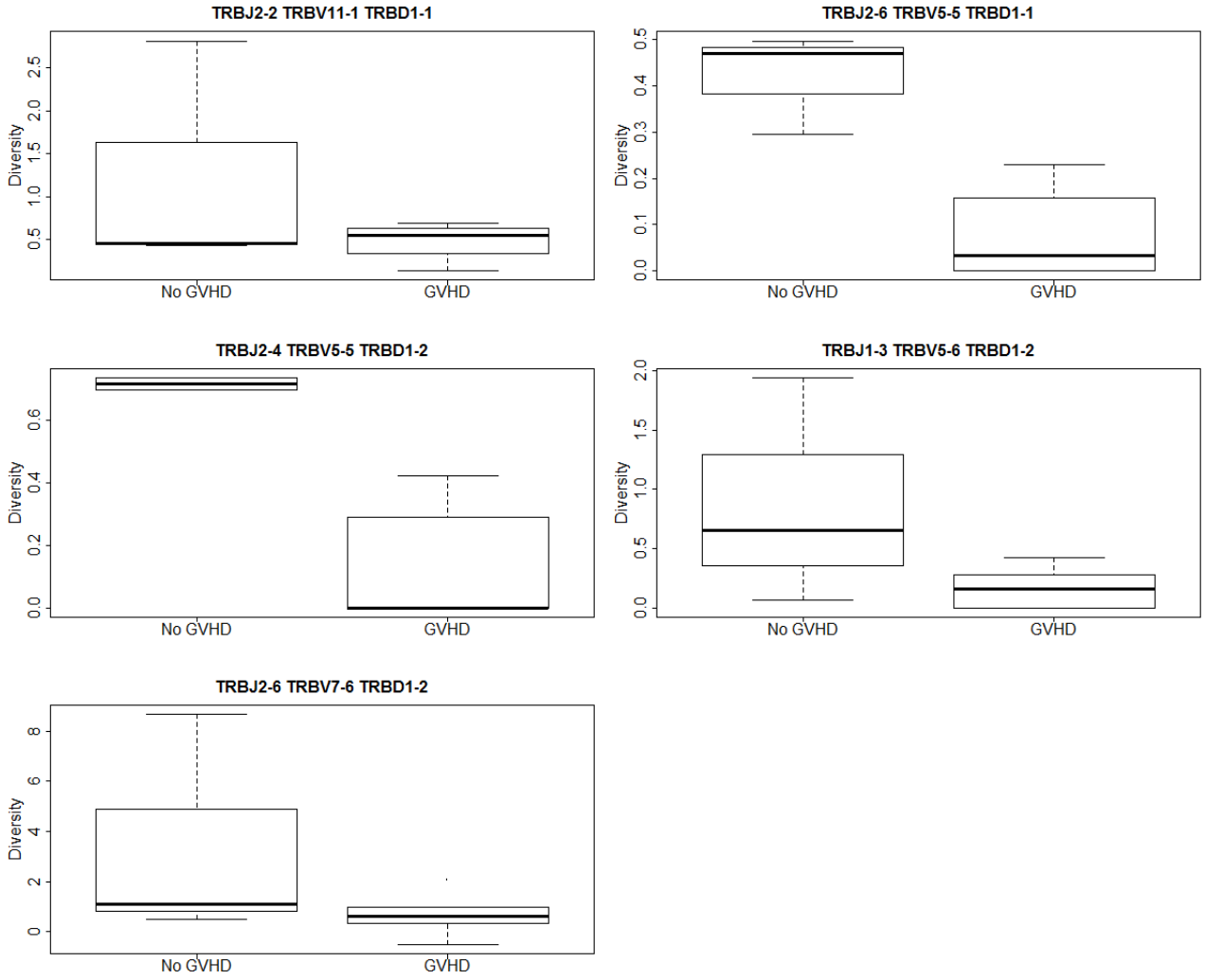


Table 4: Median Simpson's Diversity

JGene	VGene	DGene	Median No GVHD Diversity	Median GVHD Diversity	Observed FDR< 0.05
TRBJ2-2	TRBV11-1	TRBD1-1	0.458	0.544	<0.001
TRBJ2-6	TRBV5-5	TRBD1-1	0.470	0.034	<0.001
TRBJ2-4	TRBV5-5	TRBD1-2	0.715	0.000	<0.001
TRBJ1-3	TRBV5-6	TRBD1-2	0.649	0.155	<0.001
TRBJ2-6	TRBV7-6	TRBD1-2	1.088	0.619	<0.001

For comparison Shannon's diversity indices (see section 1.5.2) were also calculated for every VDJ combination in each sample. After adjusting for multiple comparisons using the Benjamini and Hochberg FDR method, there were no significant differences (FDR $>$ 0.453). However, there were 34 p-values before adjusting below the $\alpha = 0.05$ threshold. For illustrative purposes, we examined the 6 VDJ combinations with the smallest p-values for differences between the diversity index statistic. The distribution of the statistic can be seen in Figure 14. Table 5 shows the median diversity and p-values for the 6 VDJ combinations having the smallest p-values.

Figure 14: Distribution of Shannon's Diversity Index statistic for the 6 VDJ combinations having the smallest p-values.

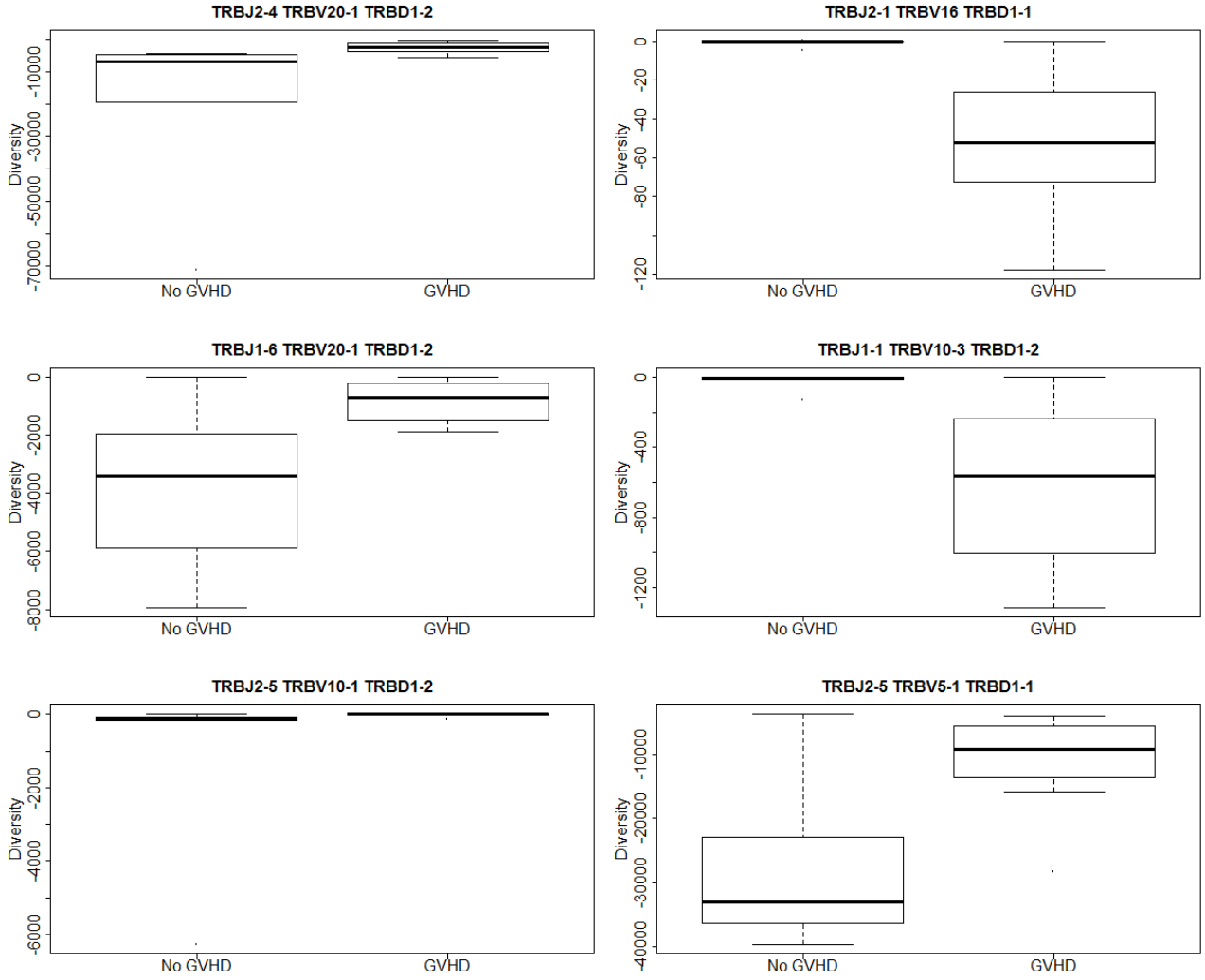


Table 5: Median Shannon Diversity

JGene	VGene	DGene	Median No GVHD Diversity	Median GVHD Diversity	p-value
TRBJ2-4	TRBV20-1	TRBD1-2	-6722.642	-2237.406	0.005
TRBJ2-1	TRBV16	TRBD1-1	>-0.001	-52.182	0.007
TRBJ1-6	TRBV20-1	TRBD1-2	-3413.701	-699.385	0.012
TRBJ1-1	TRBV10-3	TRBD1-2	-4.448	-562.484	0.013
TRBJ2-5	TRBV10-1	TRBD1-2	-100.164	-2.094	0.014
TRBJ2-5	TRBV5-1	TRBD1-1	-33045.878	-9146.991	0.015

2.8 Kullback-Leibler Divergence Results

The Kullback-Leibler (KL) Divergence method described in section 1.6.4 was adapted for TCR sequencing data and applied to the study data. Each possible VDJ combination, f, was examined individually. There were 947 F-statistics calculated using equation 31 and the K-L divergence was calculated using equation 20. Each CDR3 length, i, was considered that ranged from 1 to L. L could vary for each VDJ combination. For the equation, $n. = 13$ was the total number of subjects and $G = 2$ represents the GVHD and no GVHD groups. Further, $\Sigma_k D(\bar{\mathbf{p}}_{k.}; \bar{\mathbf{p}}_{..})$ is the within group divergence and $\Sigma_{jk} D(\mathbf{p}_{jk}; \bar{\mathbf{p}}_{k.})$ is the between group divergence. The K-L divergence was calculated using equation 20 and p_i is the overall relative abundance of CDR3 length i. Group differences were tested using equation 31. After adjusting for multiple comparisons using the Benjamini and Hochberg FDR method, there were 947 significant differences. This is all possible differences so only the lowest p-values were looked at graphically. As Figures 15 and 16 show, there is considerable variability within a group in the CDR3 length distribution. Thus, an average distribution would not be very informative in this particular data set.

Figure 15: CDR3 length distribution for each patient of VDJ combination: TRBJ2-1, TRBV18, TRBD1-1.

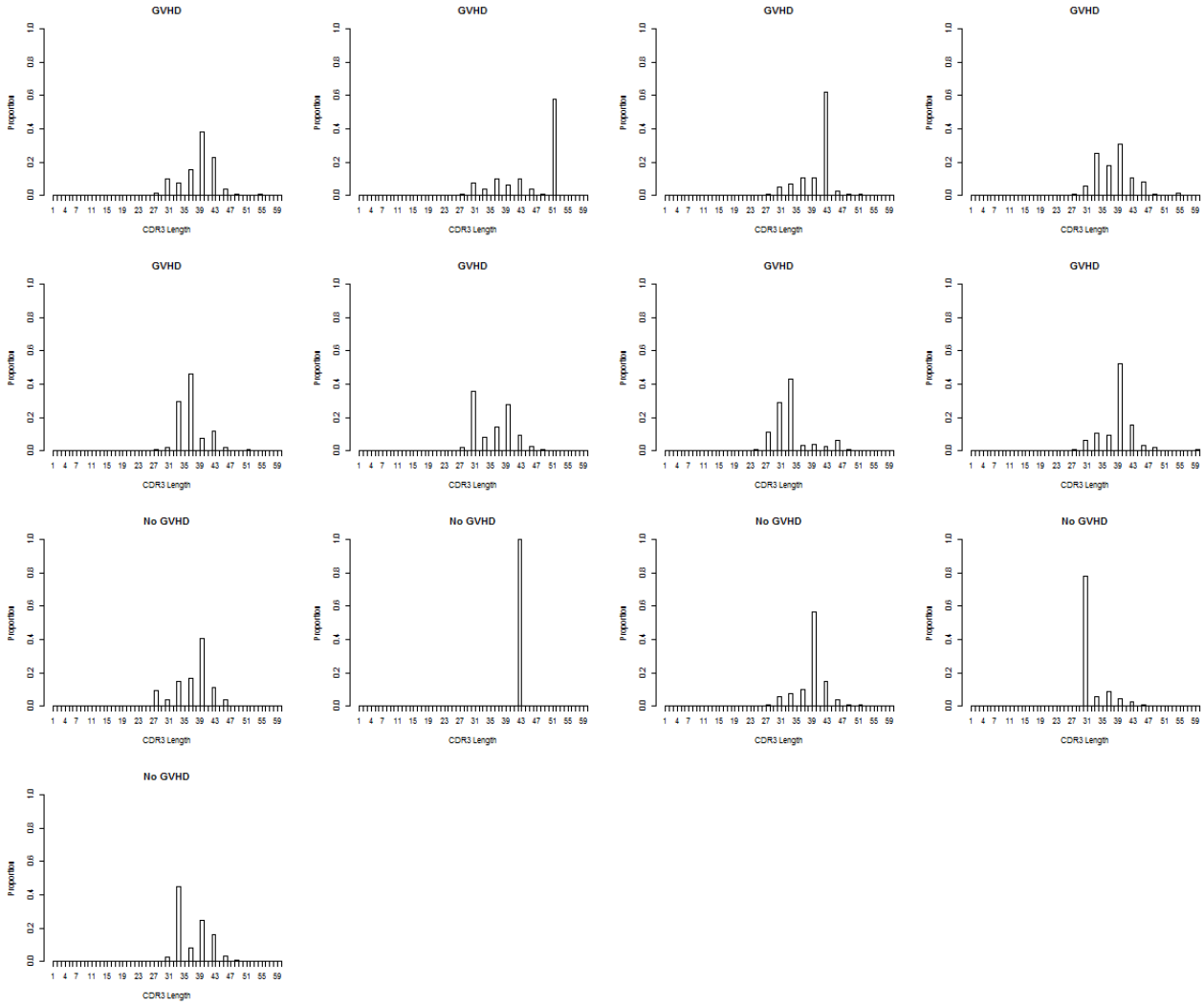
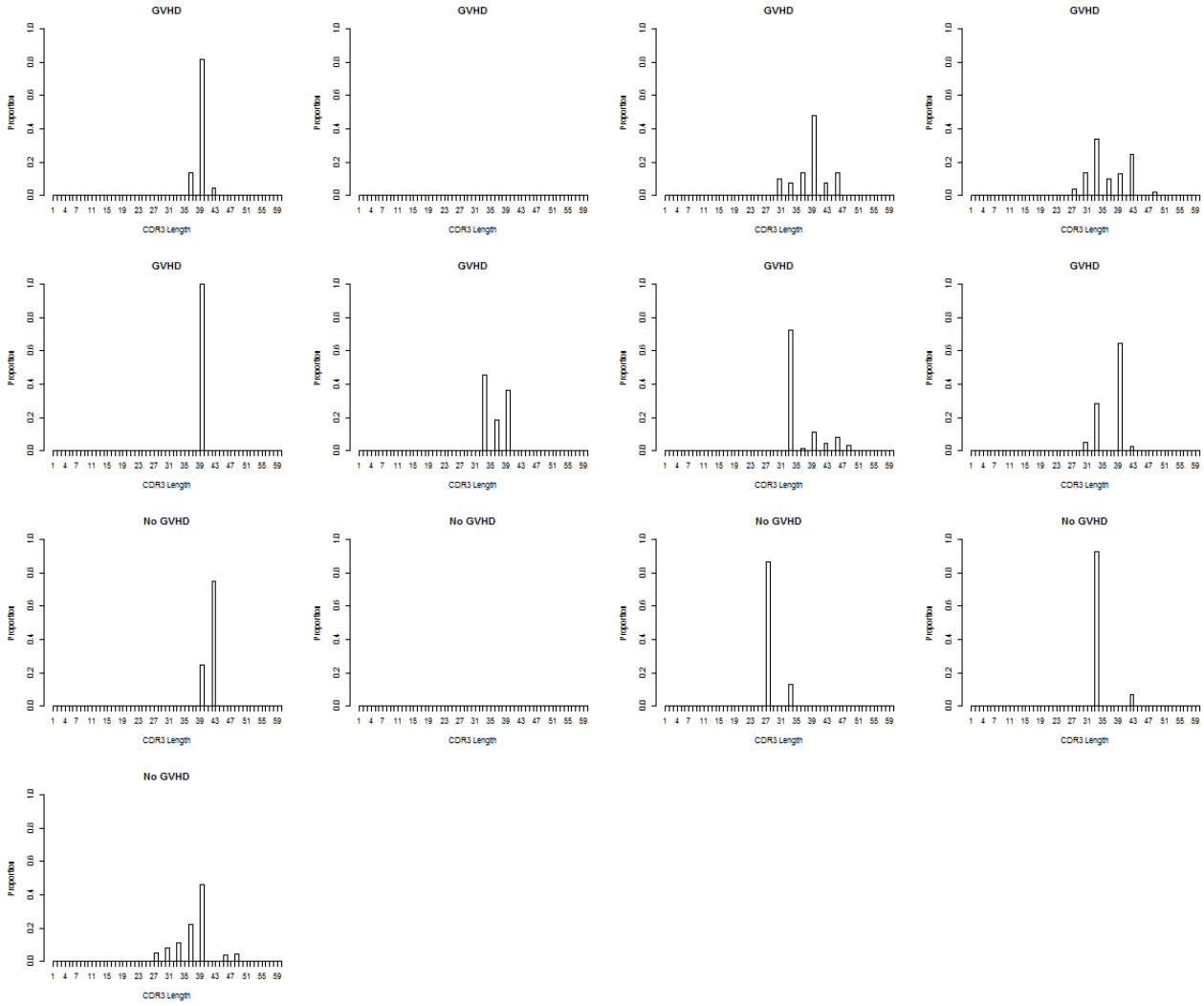


Figure 16: CDR3 length distribution for each patient of VDJ combination: TRBJ1-2, TRBV3-1, TRBD1-1.



2.9 Non-parametric Method Results

The non-parametric method described in section 1.6.5 was adapted for TCR sequencing data and applied to the study data. Each possible VDJ combination, f, was examined individually. There were 947 α values calculated for each subject, k. Each CDR3 length, i, was considered that ranged from 1 to L. L could vary for each VDJ combination. First, standard deviation was calculated for each CDR3 length, i, using equation 33, where the

probability of each CDR3 length was calculated by summing the RPM for each unique CDR3 length and then dividing by the total RPM present within the VDJ combination of interest. Only one step of variation was considered, the final TCR sampling step, so $S = 1$ and equation 34 was used to calculate the standard deviation. This standard deviation was then used to estimate the complementary cumulative frequency distribution from equation 32. An α was then calculated using equation 37 by setting $p = (0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9)$ and taking the log of both sides to estimate a slope. Permutation tests described in section 2.4 were used to test for differences between the α values of the GVHD and no GVHD groups.

The α values were calculated for every VDJ combination in each sample. After adjusting for multiple comparisons using the Benjamini and Hochberg FDR method, there was one significant difference (FDR < 0.001). The distribution of the statistic for the significantly different VDJ combination can be seen in Figure 17. Table 6 shows the median α values for the VDJ combination and the observed FDR.

Figure 17: Distribution of the non-parametric α statistic for the significantly different VDJ combination.

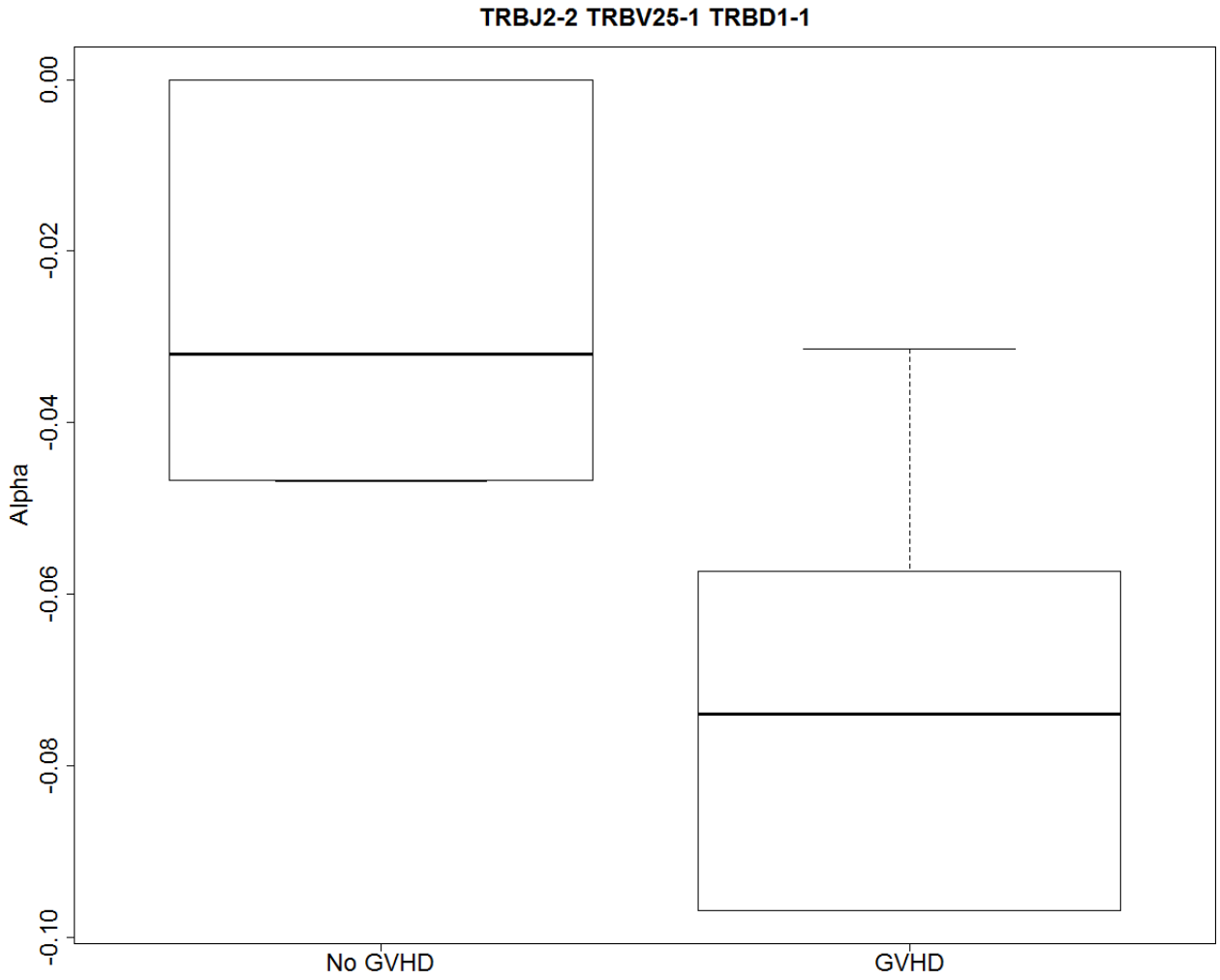


Table 6: Median Alpha Value

JGene	VGene	DGene	Median No GVHD Alpha	Median GVHD Alpha	Observed FDR< 0.05
TRBJ2-2	TRBV25-1	TRBD1-1	-0.032	-0.074	<0.001

2.10 Proportion Logit Transformation

The procedure described in section 1.6.6 was applied to the study data. Recipient data was matched to donor data by amino acid sequences. Proportions for shared sequences were then calculated for each VDJ combination and logit transformed. Because logit transformed proportions are assumed to follow a Gaussian distribution, Student's t-tests were applied to each VDJ combination to test for difference between the GVHD and no GVHD groups. After adjusting for multiple comparisons using the Benjamini and Hochberg FDR method, there were no significant differences found (FDR > 0.659). However, there were 2 p-values before adjusting below the $\alpha = 0.05$ threshold. For illustrative purposes, we looked at the 2 VDJ combinations with the smallest p-values for differences between the proportions. The distribution of the logit transformed proportions can be seen in Figure 18. Table 7 shows the median logit transformed proportion values for each group and the corresponding p-values.

Figure 18: Distribution of the logit transformed proportion of shared sequences for the 2 VDJ combinations having the smallest p-values.

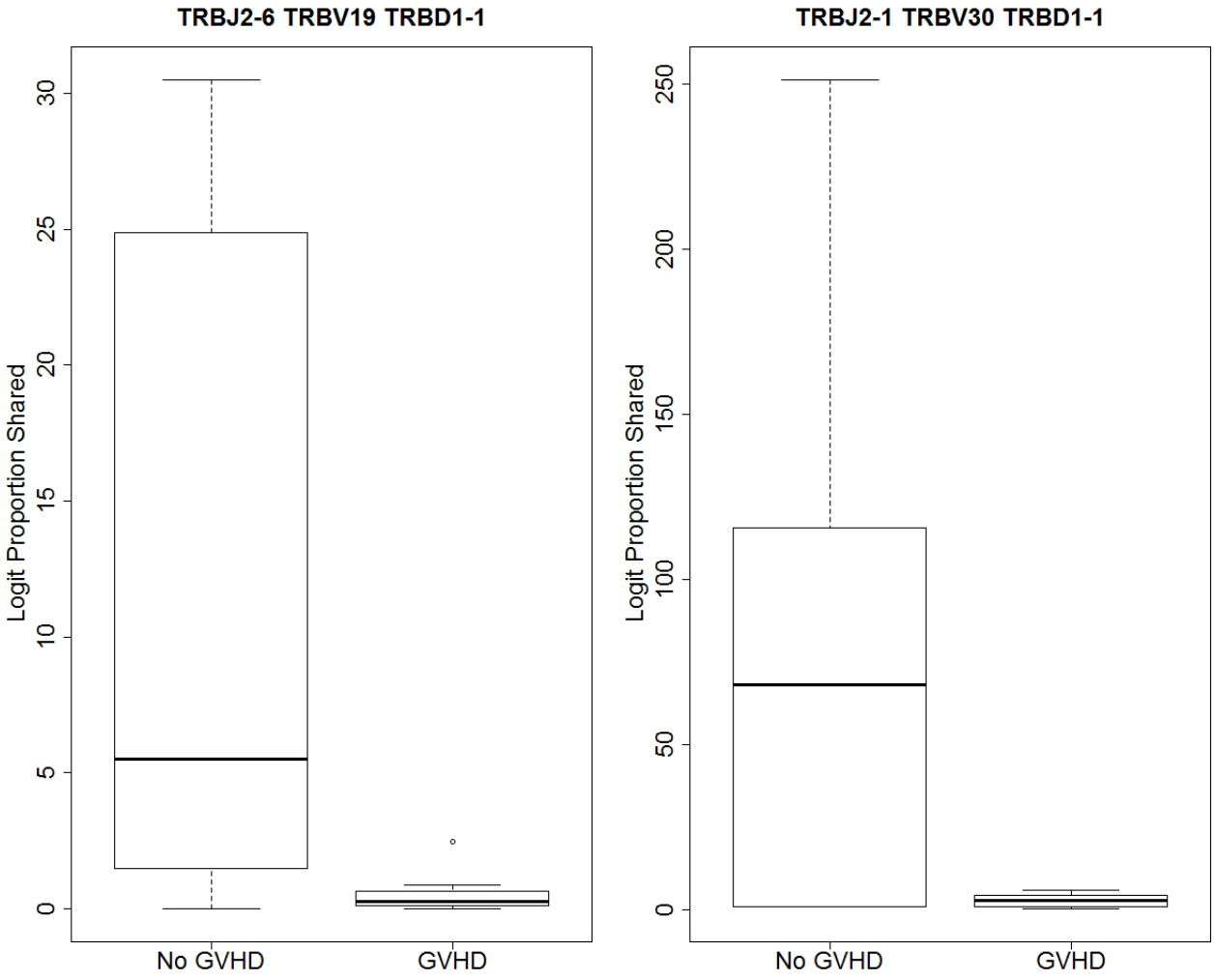


Table 7: Median of the logit transformed proportion of shared sequences

JGene	VGene	DGene	Median No GVHD Proportion	Median GVHD Proportion	p-value
TRBJ2-6	TRBV19	TRBD1-1	5.496	0.271	0.033
TRBJ2-1	TRBV30	TRBD1-1	68.000	2.748	0.037

2.11 Discussion

This chapter looked at several different methods to analyze TCR data. Permutation tests were then used to compare GVHD and no GVHD groups. Four methods identified several significantly different VDJ combinations after adjusting for multiple comparisons. None of the these methods found the same significant VDJ combinations. This could be because the methods attempt to quantify different aspects of the T-cell repertoire. The CDR3 perturbation method measures either how different the recipient distributions are from the donor or from a control distribution. On the other hand, the diversity measure simply describes how diverse the recipient's repertoire is at that point in time. Oligoscores simply look for major peaks within groups. The Kullback-Leibler divergence measures difference in distributions but assumes an underlying distribution so appears to be more sensitive to changes than the perturbation method. The non-parametric method assumes the distribution follows a power law and can be interpreted as a measure of diversity. Finally, the proportion method compares patient and donor similarity. Some of these methods measure similar aspects of the repertoire while some measure very different aspects. So the method that bests corresponds to the research question should be chosen. For this data set, Simpson's diversity index would be an appropriate choice since T-cell diversity has been shown to be a good measure of T-cell repertoire health.

3 Compositional Data in Bioscience

In the previous chapters several different methods were applied to high-throughput sequencing data. However, none of these methods consider the unit sum constraint on the data. This constraint can have large effects on data analysis and data of this type is called compositional. This chapter will describe what effects compositional data can have on analysis and how to analyze compositional data.

3.1 Introduction

To motivate what compositional data are and how they can affect data analyses consider two scenarios that Pawlowsky-Glahn and Buccianti [2011] described in their textbook on compositional data analysis. The first scenario pertains to counting the number of different messenger RNAs (mRNA) that emerge from the nucleus of a cell in a given time interval. The production of one type of mRNA does not necessarily affect the production of another mRNA. This means each mRNA can be considered independent of another mRNA and traditional analytical approaches are appropriate. The second scenario pertains to counting total mRNA within a cell that has reached its maximum capacity of mRNA. If one type of mRNA increases within the cell then another mRNA must decrease by some mechanism such as degradation. This is the sum-constrained property of compositional data that can have large effects on both the information gleaned and the interpretation of results.

There are quite a large amount of compositional data being generated in using "omics" technologies (genomics, transcriptomics, etc.) but little awareness to the fact that these are compositional data and should be treated as such. A lot of ground has been gained in the geosciences in dealing with compositional data [Pawlowsky-Glahn and Buccianti, 2011]. However, there are some big differences between the subject matters, the main one being that sequencing data produces compositions with hundreds-of-thousands of components whereas geoscience data usually produces tens to hundreds of components. This lower dimensionality

makes the data much easier to work with. For example, we can look at TCR sequencing data which appears to provide the absolute abundance of different TCR transcripts but, in reality, TCR sequencing provides only relative information for the specific sample. Note that depending on when and how the sample is prepared the sequencing can be dominated by several specific transcripts. This can be seen in experiments performed by van Heijst et al. [2013] in monitoring T-cell repertoire recovery after HSCT over time. In this case, one patient had a large spike in a TCR related to Epstein-Barr virus at day 145 which was undetectable at days 138 and 194. Furthermore, low frequency transcripts can be highly affected by changes in high frequency transcripts because only a limited number of transcripts can be sequenced. Even worse, if an experiment is repeated, the subset of low frequency transcripts detected can change with each run [Robins et al., 2009]. During TCR sequencing, a fixed amount of sample is procured and then a fixed amount of that sample is sequenced. The data are then "standardized"(ie reads per million) which results in a relative abundance. This constraint has not always been taken into account in current analytical practices. In the following sections, we will define compositional data and propose that compositional methods are more appropriate to apply to sequencing data, specifically TCR sequencing.

3.2 Definition of Compositional Data

Compositional data are defined as data where the elements of the composition are non-negative and sum to unity. This data structure often arises from non-negative data, such as counts, that has been scaled by the total of the components [Pawlowsky-Glahn and Buccianti, 2011]. Compositional data anlysis was most recently defined by Egozcue [2009]. The formulation he proposes is separated into a definition and three principles for a total of four parts.

Definition A compositional vector, or simply a composition, of D parts is a positive real vector of D components, describing quantitatively the parts of some whole, which carry exclusively relative information between the parts.

Principle A Scale Invariance: The information in a composition does not depend on the particular units in which the composition is expressed. Proportional positive vectors represent the same composition. Any sensible characteristic of a composition should be invariant under scale.

Principle B Permutation Invariance: Permutation of components of a composition does not alter information conveyed by the compositional vector.

Principle C Subcompositional Coherence: Information conveyed by a composition of D parts should not be in contradiction with that coming from a sub-composition containing d parts, $d \leq D$.

3.3 Representations of a Composition

To represent a composition so that the representation follows the principles in section 3.2, Aitchison [1986] proposed that all compositions be treated on a log transformed scale. This allows for most classical multivariate methods to be applied to compositional data. Log contrasts are scale invariant log ratios generically defined as

$$\sum_{i=1}^{D} \alpha_i \log(x_i), \text{ where} \Sigma_{i=1}^{D} \alpha_i = 0 \tag{40}$$

where α_i is a transformation coefficient, x_i is the compositional data and D is the size of the simplex. The condition on the coefficients guarantees scale invariance. Three different transformations are proposed [Pawlowsky-Glahn and Buccianti, 2011] for use depending on the hypothesis to be tested. The first is called the additive-log-ratio transformation (alr). If $\mathbf{x}$ is a composition in the D-part simplex $\mathcal{S}^D$,

$$alr(\mathbf{x}) = \log\left(\frac{x_1}{x_D}, \frac{x_2}{x_D}, ..., \frac{x_{D-1}}{x_D}\right) \tag{41}$$

where the natural logarithm is applied componentwise. The transformation is easily inverted to get the composition back. However, it is not invariant under permutation of components which may cause problems with some statistical tests. To remedy this problem, Aitchison [1986] proposed another transformation called the centered log-ratio transformation (clr) that is defined as

$$clr(\mathbf{x}) = \log\left(\frac{x_1}{g_m(\mathbf{x})}, \frac{x_2}{g_m(\mathbf{x})}, ..., \frac{x_D}{g_m(\mathbf{x})}\right), g_m(\mathbf{x}) = \left(\prod_{i=1}^{D} x_i\right)^{\frac{1}{D}} \tag{42}$$

where g_m is the geometric mean. However, in this case the components change when working with a subcomposition (defined in section 3.2) which is a violation of principle C. The components of a subcomposition using the clr transformation change because the geometric mean changes. Because of the sum-constrained property of compositional data, a D-part composition can only be represented with D-1 coefficients but the clr transformation tries to do this using D coefficients. The clr representation can be used as a measure of distance, called the Aitchison distance, that is discussed in more detail in section 3.4. The final representation is called the isometric log-ratio transformation (ilr) [Egozcue et al., 2003]. The major differences between the clr and ilr transformation are that the ilr transformation results in D-1 components and there are procedures that allow for this transformation to be easily interpretable. Simply, for the ilr transformation we are looking for an orthonormal basis of $\mathcal{S}^D$ using a set of compositions, $\mathbf{e_1}, \mathbf{e_2}, ...\mathbf{e_{D-1}}$ such that $\langle \mathbf{e_i}, \mathbf{e_j} \rangle_a = 0$ for $i \neq j$, and $\|\mathbf{e_i}\|_a = 1$. Where $\langle \cdot, \cdot \rangle_a$ and $\| \cdot \|_a$ are the Aitchison inner product and norm. Where $\langle \mathbf{x}, \mathbf{y} \rangle_a = \langle \text{clr}(\mathbf{x}), \text{clr}(\mathbf{y}) \rangle$ and $\|\mathbf{x}\|_a = \|\text{clr}(\mathbf{x})\|$ and $\| \cdot \|$ and $\langle \cdot, \cdot \rangle$ are the Euclidean norm and inner product respectively. Note, a full discussion of the Aitchison metric is presented in Section 3.5. Using this terminology the ilr transformation is defined as

$$ilr(\mathbf{x}) = (\langle \mathbf{x}, \mathbf{e}_1 \rangle_a, \langle \mathbf{x}, \mathbf{e}_2 \rangle_a), ..., \langle \mathbf{x}, \mathbf{e}_{D-1} \rangle_a). \tag{43}$$

3.4 Statistical Modelling

Compositional data do not lend themselves well to traditional statistical approaches. Issues such as spurious correlations can affect analytical results. For this reason some kind of transformation is necessary prior to performing statistical analysis. Further, compositional data are not part of the natural sample space and do not follow Euclidean geometry. The observations can be moved to a new sample space or represented using an orthonormal basis [Pawlowsky-Glahn and Buccianti, 2011]. The "move method" is the log-ratio transformation method and the "stay method" includes the alr, clr and ilr transformations that were mentioned in section 3.3. These transformations place the compositional data in terms of its basis and corresponding coordinates. These coordinates, based on linear algebra theory, are part of the real space and standard multivariate techniques can be applied to them.

The simplex can be defined as follows: Let $\mathbf{x} = (x_1, x_2, ..., x_D)$ denote a D-part composition, then

$$S^D = \left(\mathbf{x} = (x_1, x_2, ..., x_D) : \quad x_i > 0 \ \ (i = 1, 2, ..., D), \ \ \sum_{i=1}^{D} x_i = \kappa \right) \tag{44}$$

where κ is a positive constant that is typically 1 or 100 depending on how the composition is designed. The simplex has two basic operations called perturbation and powering as its internal and external operations.

$$\mathbf{x} \oplus \mathbf{x}^* = \mathcal{C}(x_1 x_1^*, ..., x_D x_D^*), \tag{45}$$

$$\alpha \odot \mathbf{x} = \mathcal{C}(x_1^\alpha, ..., x_D^\alpha), \tag{46}$$

where $\mathcal{C}$ denotes the closure operation that normalises any vector to a constant sum. Furthermore, we can define an inner product, norm and distance to define a (D-1) Euclidean vector space structure on the simplex;

$$\langle \mathbf{x}, \mathbf{x}^* \rangle_a = \frac{1}{D} \sum_{i<j} \log \frac{x_i}{x_j} \log \frac{x_i^*}{x_j^*}; \tag{47}$$

$$\| \mathbf{x} \|_a = \sqrt{\frac{1}{D} \sum_{i<j} \left(\log \frac{x_i}{x_j} \right)^2}; \tag{48}$$

$$d_a(\mathbf{x}, \mathbf{x}^*) = \sqrt{\frac{1}{D} \sum_{i<j} \left(\log \frac{x_i}{x_j} - \log \frac{x_i^*}{x_j^*} \right)^2} \tag{49}$$

Note that these equations are applied to the original simplex and are called the Aitchison geometry, hence the a subscript.

An appropriate basis for the simplex needs to be determined. Because compositions are a subset of the real space they can be expressed in terms of the canonical basis. However, this does not follow Euclidean space rules. Egozcue et al. [2003] suggested one orthonormal basis $\{\mathbf{e_1}, \mathbf{e_2}, ..., \mathbf{e_{D-1}}\}$;

$$\mathbf{e_i} = \mathcal{C} \left(\exp \left(\frac{1}{\sqrt{i(i+1)}} \right), ..., \exp \left(\frac{1}{\sqrt{i(i+1)}} \right), \exp \left(-\sqrt{\frac{i}{i+1}} \right), 1, ...1 \right) \tag{50}$$

and coordinates

$$\mathbf{x} = (y_1 \odot \mathbf{e_1}) \oplus (y_2 \odot \mathbf{e_2}) \oplus ... \oplus (y_{D-1} \odot \mathbf{e_{D-1}}), \tag{51}$$

with

$$y_i = \frac{1}{\sqrt{i(i+1)}} \log \left[\frac{x_1 x_2 ... x_i}{(x_{i+1})^i} \right], i = 1, 2, ..., D-1. \tag{52}$$

Once the basis is selected typical multivariate methods can be applied such as Principal Component Analysis.

3.5 Current Practices and Their Pitfalls

TCRβ sequencing data are multivariate. Most methods applied to sequence data are univariate, such as the t-tests (moderated), with extra procedures that adjust for multiple-testing. However, the effect of the sum constraint on univariate analysis results is questionable. Consider a simple thought exercise and imagine how the sum constraint can affect univariate statistics. Specifically, for a simple two-part composition, $\mathbf{x} = (x_1, x_2)$ derived from a basis $\mathbf{w} = (w_1, w_2)$ through its closure:

$$\mathbf{x} = C(\mathbf{w}) = (\frac{w_1}{w_1 + w_2}, \frac{w_2}{w_1 + w_2}).$$

Consider two different scenarios that have the same result. First, consider what is necessary for w_2 to double x_2 if w_1 is fixed,

$$\mathbf{w}' = (w_1, w_2')$$

$$\mathbf{x}' = (x_1', x_2') = (x_1', 2x_2)$$

$$\therefore \frac{w_2'}{w_1 + w_2'} = 2 \cdot \frac{w_2}{w_1 + w_2}$$

$$w_2' = 2w_2 \cdot \frac{w_1}{w_1 - w_2}$$

$$\approx 2w_2, \text{ if } w_1 >> w_2$$

Next, consider what is necessary for w_1 if we fixed w_2 to double x_2.

$$\mathbf{w}' = (w_1', w_2)$$

$$\mathbf{x}' = (x_1', x_2') = (x_1', 2x_2)$$

$$\therefore \frac{w_2'}{w_1' + w_2} = 2 \cdot \frac{w_2}{w_1 + w_2}$$

$$w_1' = \frac{1}{2}(w_1 - w_2)$$

$$\approx \frac{1}{2}w_1, \text{ if } w_1 >> w_2$$

This example demonstrates that there are two different mechanisms that can result in a doubling of x_2, but we can not determine from the data alone which mechanism produced such a doubling. When working with a composition that consists of more than two parts, such as TCRβ sequencing, x_1 can be considered as an aggregation of everything except x_2. In this scenario, "$x_2 \to kx_2$" implies $w_2 \to kw_2$" only if certain conditions apply:

1. x_2 is a relatively small component
2. the rest of the basis stays the same
3. $\log k$ is small

These three conditions are what, in practice, occur in 'spike-in' experiments but not necessarily in sequencing experiments. 'Spike-in' experiments are when a known concentration is added to an unknown mixture usually to assess assay sensitivity. x_2 represents the 'spike-in' portion of the sample and is usually small compared to the rest of the sample. The known mixtures are tested while the rest of the sample stays unchanged. In this case, it is safe to use univariate statistics since the compositions are not changing dramatically and follow the three conditions above. It can be assumed that samples in sequencing experiments are much

more variable than in controlled 'spike-in' experiments. Thus, using these kinds of experiments as proof of concept to be applied to more variable data, such as TCRβ sequencing, can be misleading.

Multivariate methods are commonly applied in the analysis of high-throughput data. Specifically, clustering methods are applied to identify homogeneous groups. Clustering methods require that a distance measure be calculated between samples. Lovell et al. [2010] described four different distance metrics that can be used as measures of distance for multivariate methods: the Aitchison distance, Euclidean distance, the Euclidean distance between log values, and the Kullback-Leibler divergence. Knowing how these metrics behave can help select the appropriate metric for analysis.

Consider two compositions, $\mathbf{x}$ and $\mathbf{y}$, with positive components x_i and y_i, where $i = 1, ...D$ and D is the number of components of the composition. For TCRβ sequencing the D components are the unique nucleotide sequences, unique amino acid sequences or unique CDR3 lengths. The analysis will depend on the research question and can be done for the overall components or separated by VDJ family. Their perturbation difference, $\mathbf{z} = \mathbf{x} \ominus \mathbf{y}$, is defined by

$$\mathbf{z} = \mathcal{C}(\frac{x_1}{y_1}, \frac{x_2}{y_2}, ..., \frac{x_D}{y_D})$$

where $\mathcal{C}$ is the closure.

The Aitchinson distance can be written as

$$d_a(\mathbf{x}, \mathbf{y}) = \sqrt{\| \ clr(\mathbf{x}) - clr(\mathbf{y}) \ \|} \tag{53}$$

$$= \sqrt{\| \ clr(\mathbf{z}) \ \|} \tag{54}$$

$$= \sqrt{\sum_i (\log \frac{x_i}{g_m(\mathbf{x})} - \log \frac{y_i}{g_m(\mathbf{y})})^2} \tag{55}$$

$$= \sqrt{\sum_i (\log \frac{z_i}{g_m(\mathbf{z})})^2} \tag{56}$$

where g_m is the geometric mean. By writing the Aitchinson distance in terms of $\mathbf{z}$, it can be seen that the Aitchinson distance is a measure of relative distances. In comparison, the Euclidean distance between $\mathbf{x}$ and $\mathbf{y}$ is written as

$$d_e(\mathbf{x}, \mathbf{y}) = \sqrt{\sum_i (x_i - y_i)^2} \tag{57}$$

$$= \sqrt{\sum_i (\frac{x_i}{y_i} - 1)^2 y_i^2} \tag{58}$$

Notice that this distance depends not only on the ratio of the two compositions but on one of the compositions itself. Also, the Euclidean distance is bounded by $\sqrt{2}$ whereas the Aitchison distance has no upper limit. The Aitchison distance would do a much more appropriate job because it is not constrained by an upper limit and can give more information about relative differences. Further, the Aitchinson distance is not constrained to lie on a simplex and does not have a sum-constraint.

High-throughput genomic data are frequently $\log_2$ transformed. For illustrative purposes Pawlowsky-Glahn and Buccianti [2011] showed how the Aitchison distance compares to the Euclidean distance between logged values. The Euclidean distance between log transformed values is

$$d_e(\log(\mathbf{x}), \log_2(\mathbf{y})) = \sqrt{\sum_i (\log(x_i) - \log(y_i))^2} \tag{59}$$

$$= \sqrt{\sum_i \left(\log(\frac{x_i}{g_m(\mathbf{x})}) - \log(\frac{y_i}{g_m(\mathbf{y})}) + \log(\frac{g_m(\mathbf{x})}{g_m(\mathbf{y})}) \right)^2} \tag{60}$$

$$= \sqrt{d_a^2(\mathbf{x}, \mathbf{y}) + D \log^2 \left(\frac{g_m(\mathbf{x})}{g_m(\mathbf{x})} \right)} \tag{61}$$

$$\geq \sqrt{d_a^2(\mathbf{x}, \mathbf{y})} \tag{62}$$

This is very similar to Aitchinson's distance with an extra component, $D \log^2 \left(\frac{g_m(\mathbf{x})}{g_m(\mathbf{x})} \right)$. Because high throughput sequencing data are typically $\log_2$ transformed, similar results should be observed when comparing analyses performed using Aitchison's distance and the $\log_2$ transformed Euclidean distance [Lovell et al., 2010].

Finally, the Kullback-Leibler (K-L) divergence, which is not specifically a distance measure [Lovell et al., 2010], is typically used to measure the difference between two probability distributions. Distributions are similar to compositions in the sense that they sum to 1. Therefore, the K-L divergence is another method that can be used when comparing compositions. The K-L divergence between compositions depends on the ratios of the composition components, their absolute values of components, and the dimensionality of the compositions. Recall that the K-L divergence (see Section 1.6.4) between discrete probability distribution P and probability distribtution Q, both indexed by $i = 1$ to D where D is the size of the simplex, is

$$D_{KL}(P|Q) = \sum_i P(i) \log \frac{P(i)}{Q(i)} \tag{63}$$

The effect that compositional data has on estimates of covariance and correlation should be used to guide the decision of which metric to use. Lovell et al. [2010] showed the relationship between the log of two components in a composition. Consider two basis vectors

and their closures

$$\mathbf{w} = (w_1, w_2)$$

$$\mathbf{W} = (W_1, W_2)$$

$$\mathbf{x} = \mathcal{C}(\mathbf{w})$$

$$\mathbf{X} = \mathcal{C}(\mathbf{W}).$$

The following result will also be used

$$\text{cov}(A + C, B + C) = \text{cov}(A, B) + \text{cov}(A, C) + \text{cov}(B, C) + \text{var}(C).$$

Applying this result to the log of the compositions and the log of the basis we get

$$\begin{aligned}
\text{cov}(\log x_1, \log x_2) &= \text{cov}(\log(\frac{w_1}{t}), \log(\frac{w_2}{t})) \\
&= \text{cov}(\log w_1 - \log t, \log w_2 - \log t) \\
&= \text{cov}(\log w_1, \log w_2) - \text{cov}(\log w_1, \log t) - \text{cov}(\log w_2, \log t) + \text{var}(\log t)
\end{aligned}$$

where t is the size of the basis. Thus, the covariance of the log of two components of interest in a composition is equal to the covariance between the log of those components in the basis plus or minus some terms related to the size of the basis. If the terms related to the size of the basis are large, the covariance estimates could become very different between using the components and the basis. Similarly, since correlations are functions of variance and covariance they can also be very different between compositional data and those in the

basis. By definition the correlation is defined as

$$\text{corr}(A+C, B+C) = \frac{\text{cov}(A+C, B+C)}{\sqrt{\text{var}(A+C)}\sqrt{\text{var}(B+C)}}$$

Unfortunately, there is no nice representation of correlation to show how the components and the basis are related.

3.6 Treatment of Zeros in Compositional Data

As previously described, compositional data analysis is performed using a log ratio scale. Therefore, consideration must be given with respect to how to appropriately handle observed zeros. Aitchison et al. [2003] identified three processes that may give rise to observed zero values. The process that gave rise to the zero values should guide the analyst as to how to deal with the zero values. First, a zero value may truly represent a zero in the data. This is known as a structural zero or essential zero. Second, a zero might occur because of an underlying discrete process such as space on a sequencing chip. For example, in TCR sequencing data, CDR3 sequences with low counts may not be sequenced because clonotypes with high frequency used up all the space on the chip. Third, zeroes can occur because values fall below the limit of detection.

Rounded zeros are zeros when very small values are rounded to zero because of rounding error or the value falls below the limit of detection. In compositional data, these zeros fall under the category of not missing at random (NMAR) and require special models. Generally, methods for working with rounded zeroes can be classified into either non-parametric or parametric techniques. The non-parametric methods are based on different imputation methods. On the other hand, the parametric methods rely on parametric models such as the normal distribution on the simplex.

Counts are values that occur when the number of times an event occurs is measured. In the case of TCR sequencing data the number of times a particular CDR3 sequence occurs is

being counted. Since TCR sequencing data are transformed into RPM (reads per million), a measure of relative abundance, they should be considered in the compostional framework. Pierotti et al. [2009] suggest a Bayesian-multiplicative approach.

The multiplicative part of the approach is based on an imputation strategy for rounded zeros [Martín-Fernández et al., 2003]. This strategy involves replacing the zeros with an appropriately small value δ_j and then modifying the non-zeros in a multiplicative way, where $j = 1, ..., n$ the number of observations. The specific modification is based on the sum constraint for imputation data. Let us consider a D-composition, $\mathbf{x} \in S^D$ that has rounded zeros. This composition is replaced by a new composition $\mathbf{r} \in S^D$ according to the formula

$$r_j = \begin{cases} \delta_j & \text{if } x_j = 0 \\ x_j \left(1 - \frac{\sum_{k|x_k=0} \delta_k}{c}\right) & \text{if } x_j > 0 \end{cases} \tag{64}$$

where c is the constant for the sum constraint and δ_j is a chosen value below the limit of detection. This method mixed with a Bayesian approach can be used to impute zero values in count data.

Now let $\mathbf{x}$ be a count vector with D categories in data set $\mathbf{X}$. Let N be the total count in $\mathbf{x}$ and θ be its vector of probabilities from a multinomial distribution. Then the prior distribution for θ is a Dirichlet iid parameter vector α where $\alpha_k = st_k$, where $k = 1....D$. The vector $\mathbf{t}$ is the prior expectation for θ and s is known as the strength of that prior. The parameter s can be chosen to be between 0 and 1. From Bayes theorem the posterior estimation for θ is

$$\hat{\theta} = \frac{x_k + st_k}{N + s} \tag{65}$$

There are several different proposed priors but all come from the Dirichlet distribution. Then the chosen prior can be used to apply the multiplicative part of the method to actually impute zero values using a modification of the rounded zeros formula

$$r_j = \begin{cases} \frac{\alpha_j}{N+s} & \text{if } x_j = 0 \\ x_j \left(1 - \sum_{k|x_k=0} \frac{\alpha_j}{N+s}\right) & \text{if } x_j > 0 \end{cases} \tag{66}$$

In this way we are able to preserve the ratios of the non-zero values while having a data set that contains no zeros. This new data set also preserves the unit constraint.

3.7 Application of Compositional Methods to TCR data

The data described in section 2.3 were treated as compositional data. All compositional calculations were done using the coin package in the R programming environment [Zeileis et al., 2008]. First each VDJ combination was treated as one part of a 947 part composition. The top 10 % most variable VDJ combinations were considered for clustering. Clustering was then done using the Aitchison distance and Ward's minimum variance method. Figure 19 shows the results of the clustering. Notice that other than the 2 No GVHD patients there seems to be no obvious grouping pattern.

Next, the CDR3 length distribution within each VDJ combination was considered as an L-part compositions. To compare groups linear models of the following form were run

$$\mathbf{Y}_i = \mathbf{a} \oplus X_i \odot \mathbf{b} + \epsilon_i \tag{67}$$

$$\text{ilr}(\mathbf{Y}_i) = \text{ilr}(\mathbf{a}) + X_i \text{ilr}(\mathbf{b}) + \text{ilr}(\epsilon_i) \tag{68}$$

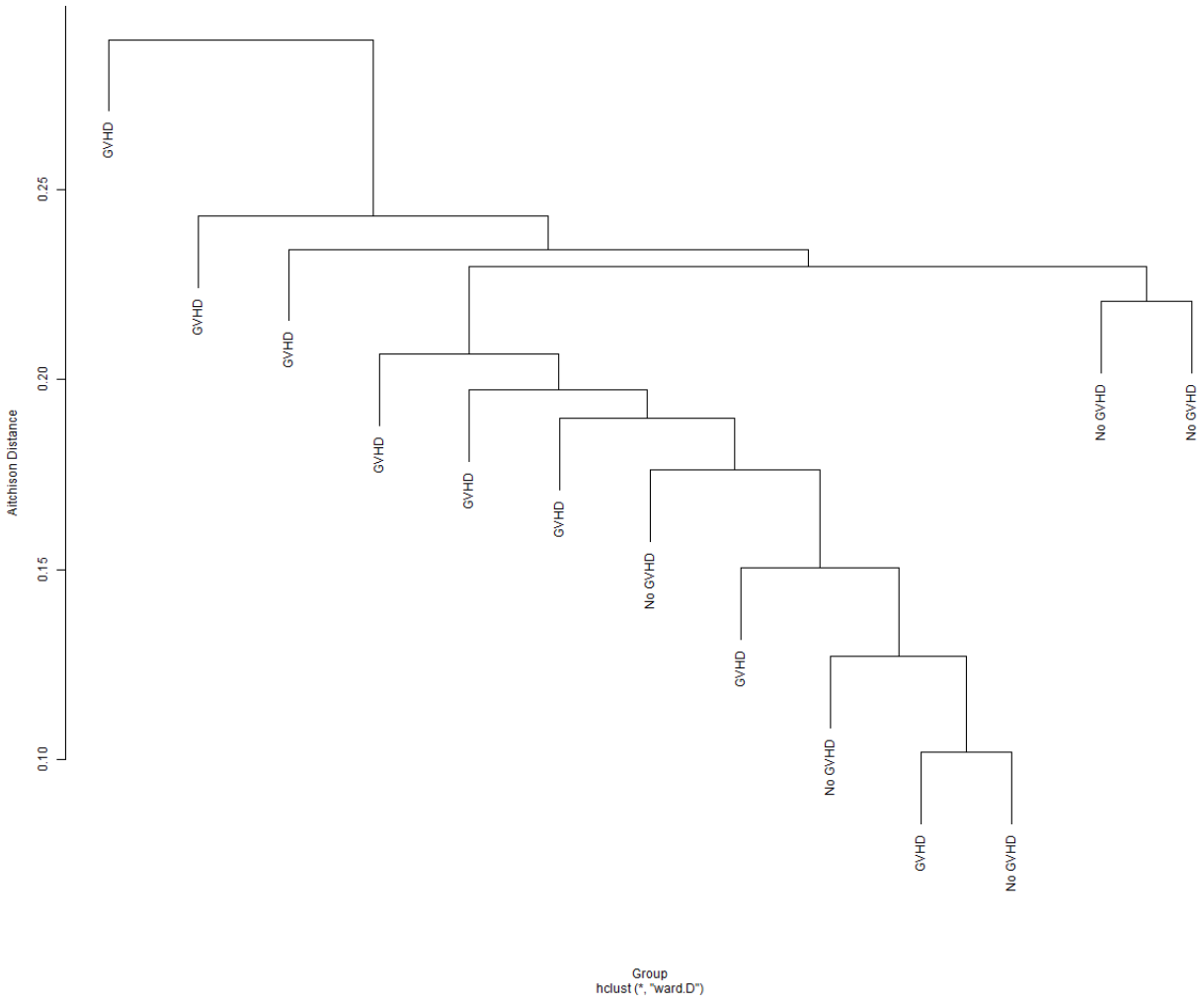
where $\mathbf{a}$ and $\mathbf{b}$ are compositional constants, $\mathbf{Y}_i$ is a random composition, X_i is the group indicator and ϵ_i is a compositional random variable with compositional expectation $(1, ..., 1)/L$ and constant variance [Zeileis et al., 2008]. There were 947 models run to compare groups at each VDJ combination using this model. Zero values were replaced by a below limit of detection value of $1e-6$. After adjusting for multiple comparisons using the Benjamini and

Hochberg FDR method, there were no significant differences found (FDR > 0.534). However, there were 20 p-values before adjusting below the $\alpha = 0.05$ threshold. Table 8 shows the 20 p-values.

Table 8: Compositional Linear Model p-values

JGene	VGene	DGene	p-value
TRBJ2-3	TRBV10-2	TRBD1-1	0.001
TRBJ2-4	TRBV5-6	TRBD1-1	0.001
TRBJ2-4	TRBV28	TRBD1-1	0.002
TRBJ2-7	TRBV11-1	TRBD1-2	0.005
TRBJ2-5	TRBV6-1	TRBD1-2	0.005
TRBJ2-3	TRBV4-2	TRBD1-2	0.006
TRBJ2-4	TRBV7-6	TRBD1-1	0.010
TRBJ2-5	TRBV27	TRBD1-2	0.014
TRBJ2-6	TRBV11-2	TRBD1-2	0.015
TRBJ2-3	TRBV28	TRBD1-2	0.019
TRBJ2-5	TRBV4-3	TRBD1-1	0.022
TRBJ2-7	TRBV27	TRBD1-2	0.022
TRBJ2-7	TRBV7-3	TRBD1-2	0.027
TRBJ2-5	TRBV13	TRBD1-1	0.029
TRBJ2-1	TRBV5-5	TRBD1-1	0.032
TRBJ2-7	TRBV14	TRBD1-1	0.033
TRBJ2-5	TRBV9	TRBD1-1	0.034
TRBJ2-5	TRBV27	TRBD1-1	0.035
TRBJ2-1	TRBV16	TRBD1-1	0.037
TRBJ2-5	TRBV28	TRBD1-1	0.049

Figure 19: Cluster Dendrogram of the 13 HSCT patients.



3.8 Discussion

In this chapter we discussed what compositional data are and why sequencing data falls in that category. The compositional methodology was then applied to the TCR sequencing data described in section 2.3. The compositional models identified different VDJ combinations compared to all the methods applied in chapter 2. This shows that choosing the appropriate analysis strategy can have large effects on results. The research question of interest in this study was to identify VDJ combinations that differ between the GVHD and no GVHD

groups. This seems to best be addressed by Simpson's Diversity measure since it has a biological interpretation that more diversity is correlated with a healthier TCR repertoire. On the other hand, tests such as the Kullback-Leibler divergance method test for the equality of individual peak distributions which is not really the research question. It was of further interest to determine if the different methods identified similar V or J genes individually instead of specific VDJ combinations. Table 9 shows a summary of V or J genes that were identified by multiple methods.

Table 9: Common V or J Gene Using Different Analyses

Gene	Individual Perturbation	Mean Perturbation	Simpson's Diversity	Compositional Analysis	Alpha Value
TRBJ2-6	X		X	X	
TRBJ1-1		X		X	
TRBJ2-1		X		X	
TRBJ2-2			X	X	X
TRBJ1-3			X	X	
TRBV7-9	X	X			
TRBV9	X	X			
TRBV7-7		X		X	
TRBV7-6			X	X	
TRBV25-1				X	X

4 Generalized monotone incremental forward stagewise method for modeling count data: Application predicting micronuclei frequency

This chapter considers count data that are left as counts and not transformed like sequencing data. Specifically, this chapter looks at a method to use high-throughput data to predict a count response. Because many methodologies cannot be applied to scenarios where there is a larger number of predictors than samples, a generalized monotone forward stagewise (GMIFS) method is considered.

4.1 Introduction

Micronuclei (MN) are small nuclear bodies that are formed in cells that are in the process of division but are not part of the nucleus. Therefore MN can only be found in cells that have undergone nuclear division at least once and appear as small extranuclear bodies. When two daughter nuclei are formed during cell division, these bodies are placed into a smaller nucleus not part of the main nuclei, hence the term micronuclei [Kirsch-Volders et al., 2011]. Once the micronuclei are formed the cell has several different response options. MN can stay within the cell, if it has functional DNA, as its own entity or be reabsorbed into the main nucleus. If the DNA is non-functional the MN may be expelled from the cell or the whole cell may be destroyed through apoptosis. Since MN can be expelled from the cell, they can be used as a mechanism to remove extra chromosomes from the cell [Kirsch-Volders et al., 2011].

Micronuclei can form spontaneously or they can be induced by mutagens. Some spontaneous MN are actually beneficial to the organism. An example is in the mouse cerebral cortex where MN formation adds diversity to the nervous system [Kirsch-Volders et al., 2011]. However, the large majority of MN are caused by mutagens and may play a role in carcino-

genesis. Depending on the fate of the MN, the end result could be a variety of different DNA and chromosome cell contents. This variety could result in an accumulation of DNA changes and instability that could result in cancer [Kirsch-Volders et al., 2011]. Several studies have shown that higher MN counts result in a higher risk of cancer in the future [Kirsch-Volders et al., 2011]. Thus, using the cytokinesis-block micronucleus (CBMN) assay as a risk assessment for cancer has potential clinical benefits. Further, combining CBMN with other high-throughput technologies such as gene expression and methylation analyses may help identify factors related to micronucleation.

Quantifying MN in patient samples has been shown to be a good measure of genetic damage. MN scoring, counting the number of MN present in a sample, is a popular tool for testing genotoxicity mostly because of its simplicity, accuracy, applicability to different cell types and ease of automation. Cancer cells show a loss of genetic control which can be caused by DNA damage so they are a good candidate for MN testing. The CBMN assay has successfully been used and validated to score MN. The CBMN assay uses Cytochalasin-B which stops cells from performing cytokinesis but does not stop nuclear division giving rise to cells that are binucleated [Fenech, 1993, Fenech et al., 1999]. Furthermore, the Organisation for Economic Co-operation and Development (OECD) has developed a set of guidelines for running the CBMN assay to have the most consistent and reliable results [Kirsch-Volders et al., 2011].

Guidelines for the process of scoring MN have been presented by the HUman Micron-Nucleus (HUMN) project. This is an international collaborative project aimed at improving the application of the CBMN assay. One of the HUMN projects main goals is to identify methodological variables in the scoring of the assay to minimise confounding effects [Fenech et al., 2003]. The HUMN project compiled 6583 subjects from 25 laboratories from 16 countries and looked at background MN frequency using the CBMN assay. The goal of the study was to identify variables that affect the background MN frequency. Scoring criteria was found to account for 47% of the observed variability thus standardized scoring criterion were

developed and described by Fenech et al. [2003]. The guideline includes scoring 2000 cells in order to accurately estimate MN frequency.

Because these guidelines were developed for assay performance they do not address how to statistically analyze the data generated by the assay. This has led to the application of various statistical methods that may render different interpretations and conclusions. In a review article examing analytical methods, Ceppi et al. [2010] reviewed 63 studies that statistically analyzed MN data and developed recommendations for selecting an appropriate analytical method. The review included studies that applied both parametric and non-parametric tests. The non-parametric tests included Kruskall-Wallis, Friedman, Wilcoxon and Mann-Whitney U-tests. Although these tests do not require an underlying distributional assumption, they are unable to adjust for confounding factors. There were a variety of parametric tests performed that assume normality such as ANOVA, ANCOVA, and multivariable linear models which can adjust for confounding factors. Other methods such as correlations and Student's t-test were also used. However, applying these methods to MN data, which are rarely normally distributed, could result in inappropriate inferences. Although the data could be transformed to better adhere to a Gaussian distribution prior to applying such parametric tests, few studies applied any type of transformation. Further, Student's t-tests and Pearson's correlation cannot adjust for confounding variables. The common non-Gaussian models used were log-linear, Poisson, negative binomial and logistic regression. The logistic and log-linear models account for categories whereas Poisson and negative binomial directly model count data. For this reason Ceppi et al. [2010] recommend using negative binomial or Poisson models for MN data analysis. Another advantage of these count models is that they can adjust for confounding variables such as age, gender and smoking status. Finally, Ceppi et al. [2010] recommended 2000 or more cells be scored for best model performance. If less than 2000 cells are scored, a zero-inflated Poisson model is recommended [Ceppi et al., 2010].

When trying to identify molecular features related to MN frequency, high-throughput

genomic assays can be used. However, the previously described methods cannot be applied in settings where there are more predictor variables than samples. Therefore, in this section we extended the generalized monotone forward stagewise (GMIFS) method to the Poisson regression setting and apply it to a breast cancer study where we were interested in predicting MN frequency using features from the Illumina HumanMethylation 450K assay and to a cord blood data set where we were interested in predicting MN frequency using features from the Agilent 4x44k human oligonucleotide microarray.

4.1.1 Cytokinesis-block Micronucleus Assay

Micronuclei assays can only be effective if dividing cells can be identified. This is because MN only occur in cells that have undergone at least one nuclear division after DNA damage has occurred. Cytokinesis is the step in the cell division process that involves the splitting of the cytoplasm to form two cells. Cytochalasin-B stops cells from performing cytokinesis but does not stop nuclear division giving rise to cells that are binucleated [Fenech, 1993, Fenech et al., 1999]. However, only cells in the sample that are going to divide will take on a binucleated appearance. The sample is heterogeneous with cells that tried to divide and those that did not. Thus, there will be a mixture of mononucleated cells along with binucleated cells. Mononucleated cells in the CBMN assay can arise from several possibilities. There could be cells that never divide, cells which replicate DNA but escape nuclear division, cells that escape the cytochalasin B block or cells that are in an early stage of apoptosis or necrosis [Kirsch-Volders and Fenech, 2001]. Thus it can be considered that mononucleated cells indicate chromosome damage *in vivo*, present prior to the assay being done, and binucleated cells indicate DNA damage present before culture as well as damage expressed *in vitro* during culture. Thus, what is scored is dependent on the protocol followed and what type of hypothesis is being tested.

4.2 Methods

4.2.1 Statistical Methods

There are many available methods that can model count data. However, these methods require independence of explanatory variables (p) and that the number of samples (n) does not exceed the number of explanatory variables. The incremental forward stagewise regression method for linear regression and the Generalized Monotone Incremental Forward Stagewise (GMIFS) for a logistic regression model have been previously described [Hastie et al., 2007]. The GMIFS method for modeling ordinal response data has also been described [Archer et al., 2014]. To motivate our extension to the Poisson regression setting, we first review Poisson regression. We subsequently describe our GMIFS method for fitting Poisson regression models when $n < p$.

4.2.2 Poisson Regression

Poisson regression is commonly used to model count data. Let $i = 1, ..., n$ be the number of observations and y_i represent a Poisson distributed random variable. Let the expected value of y_i be written as

$$\mathbb{E}(y_i) = \lambda_i.$$

Then the conditional probability is given by

$$P(y_i|\lambda_i) = \frac{\mathrm{e}^{-\lambda_i}\lambda_i^{y_i}}{y_i!}$$

for each observation i. The likelihood is represented by

$$L(\lambda|\mathbf{y}) = \prod_{i=1}^{n} \frac{\mathrm{e}^{-\lambda_i}\lambda_i^{y_i}}{y_i!}.$$

Mathematically it is easier to maximize the log-likelihood which is given by

$$\ell(\lambda|\mathbf{y}) = \sum_{i=1}^{n}(y_i \log \lambda_i - \lambda_i - \log(y_i!)).$$

Thus, we are looking for the value of λ that maximizes the log-likelihood above. Further, an offset is used if the response variable can be considered a rate. For example, MN frequency is scored from a larger number of total cells. So if the total number of cells examined varies by subject an offset is appropriate. In this case the expected value is

$$\mathbb{E}(y_i) = t_i\lambda_i$$

where t_i is the offset value. The conditional probability is then given by

$$P(y_i|\lambda_i) = \frac{\mathrm{e}^{-t_i\lambda_i}(t_i\lambda_i)^{y_i}}{y_i!}$$

for each observation i. The likelihood is represented by

$$L(\lambda|\mathbf{y}) = \prod_{i=1}^{n} \frac{\mathrm{e}^{-t_i\lambda_i}(t_i\lambda_i)^{y_i}}{y_i!}.$$

Again, mathematically it is easier to maximize the log-likelihood which is given by

$$\ell(\lambda|\mathbf{y}) = \sum_{i=1}^{n}(y_i \log(t_i\lambda_i) - t_i\lambda_i - \log(y_i!)).$$

Once again we are looking for the λ value that maximizes the log-likelihood. These log-likelihoods are used to model predictor variables. In Poisson regression the model assumes that the expected value can be modeled by a linear combination of predictors. In this case the natural log of t_i is entered as an offset in the model estimation. The natural log of the expected value is

$$\log(\mathbb{E}(y_i|\mathbf{x}_i)) = \log(t_i) + \boldsymbol{\theta}'\mathbf{x}_i$$

where $\mathbf{x}_i$ is a vector of predictor variables and $\boldsymbol{\theta}$ is a vector of coefficients. The estimated coefficients can be exponentiated to determine how the response changes with the predictor. By using the estimated linear combination of coefficient estimates and taking the exponent we can calculate the estimated response of that particular subject.

4.2.3 Generalized Monotone Incremental Forward Stagewise Poisson Model

The GMIFS method was previously described for the logistic regression scenario by Hastie et al. 2007 and here we adapted it to a Poisson regression model. For the proposed method we consider three types of parameters that $\boldsymbol{\theta}$ from section 4.2.2 can be separated into along with an offset (t_i). The parameters are the intercept (α), those corresponding to an unpenalized subset of predictors ($\boldsymbol{\gamma}$), and those corresponding to a set of penalized predictors ($\boldsymbol{\beta}$). The design matrix, $\mathbf{x}$, consists of two parts, $\mathbf{x}_j$ and $\mathbf{x}_k$, where $j = 1, ..., J$ is the set of unpenalized predictors, $k = 1, ..., K$ is the set of penalized predictors and $J + K = P$ is the total number of predictors. The unpenalized predictors are those that we wish to force into the model, such as gender, age and smoking status which researchers consider important predictors of MN frequency [Ceppi et al., 2010] and their values are in the $\mathbf{x}_{ij}$ design matrix for subject i. The penalized variables (thousands of features from a high-throughput genomic experiment) are those that the model will choose for us and are considered to be the investigative predictors and their values are in the $\mathbf{x}_{ik}$ design matrix for subject i.

The algorithm proceeds in an iterative fashion and updates one of the penalized covariates by a small incremental amount at each step. To determine which penalized covariate is to be updated next the largest negative gradient is used. Thus we need to calculate the first derivative of the log-likelihood corresponding to each penalized predictor. The log-likelihood written in terms of α, $\boldsymbol{\beta}$ and $\boldsymbol{\gamma}$ is

$$\ell(\alpha, \boldsymbol{\beta}, \boldsymbol{\gamma}|\mathbf{y}, \mathbf{x}_j, \mathbf{x}_k) = \sum_{i=1}^{n} (y_i(\alpha + \log(t_i) + \boldsymbol{\gamma}\mathbf{x}_{ij} + \boldsymbol{\beta}\mathbf{x}_{ik}) \\ - \exp(\alpha + \log(t_i) + \boldsymbol{\gamma}\mathbf{x}_{ij} + \boldsymbol{\beta}\mathbf{x}_{ik}) - \log(y_i!))$$

and the first derivative written in terms of α, $\boldsymbol{\beta}$ and $\boldsymbol{\gamma}$ in matrix notation is

$$\frac{\partial \ell}{\partial \boldsymbol{\beta}} = \mathbf{x}'(\mathbf{y} - \exp(\alpha + \log(t_i) + \boldsymbol{\gamma}\mathbf{x}_j + \boldsymbol{\beta}\mathbf{x}_k)).$$

Once we know which covariate to update, we need to determine in what direction to update the covariate. In order to know the direction of the update the second derivative would need to be calculated which is a cumbersome process. Hastie et al. 2007 showed that to avoid having to calculate the second derivative an expanded covariate space can be used. Using the notation mention previously where $\mathbf{x}_j$ are the unpenalized variables and $\mathbf{x}_k$ are the penalized subset, the expanded covariate space is $\tilde{\mathbf{x}} = [\mathbf{x_j} : \mathbf{x_k} : -\mathbf{x_k}]$. For example, let $\beta_1, ..., \beta_p$ be the positive coefficient estimates and $\beta_{p+1}, ..., \beta_{2p}$ be the coefficient estimates of the negative version of $\mathbf{x}_k$. Then $\boldsymbol{\beta}$ is calculated by subtracting the pairs, $\beta_1 - \beta_{p+1}, ..., \beta_p - \beta_{2p}$. Our proposed GMIFS algorithm using the expanded covariate set is

1. Initialize the components of $\hat{\boldsymbol{\beta}}^{(s)} = 0$ at step $s = 0$.

2. Initialize the intercept α and the unpenalized coefficients γ_j where $j = 1, ..., J$ using a maximization algorithm of the log-likelihood.

3. Considering α and $\boldsymbol{\gamma}$ fixed, find the predictor $\mathbf{x}_m$ where $m = \underset{2K}{\operatorname{argmin}} \left(-\frac{\partial \ell}{\partial \beta_k}\right)$ at the current estimate $\hat{\boldsymbol{\beta}} = \hat{\boldsymbol{\beta}}^{(s)}$.

4. Update the corresponding coefficient $\hat{\beta}_m^{(s+1)} = \hat{\beta}_m^{(s)} + \epsilon$ to yield a new vector of parameter estimates.

5. Update α and the unpenalized coefficients, γ_j, by maximum likelihood considering the $\hat{\boldsymbol{\beta}}^{s+1}$ from step 4 as fixed.

6. Repeat steps 3-5 until the difference between successive log-likelihoods is less than a pre-specified tolerance, τ. The defaults for the GMIFS algorithm are $\epsilon = 0.001$ and $\tau = 0.00001$.

4.2.4 Comparative Method: Penalized Linear Regression

A penalized linear regression model can be fit by adding a penalty term to the sums of squares. Specifically, the `glmpath` algorithm uses a linear combination of the L_1 and L_2 norm penalizations. The `glmpath` algorithm is based on a previous algorithm called LASSO. LASSO minimizes the typical sum of squares with an added constraint. Specifically for linear regression LASSO minimizes [Tibshirani, 2011]

$$\sum_{i=1}^{N}(y_i - \sum_{j} x_{ij}\beta_j)^2 + \lambda \sum_{j=1}^{p} |\beta_j|$$

where x_{ij} are the standardized predictors and y_i is the set of centered responses for $i = 1, ..., N$ and $j = 1, ..., p$. Because of the form of the constraint, LASSO does both variable selection and shrinkage. The `glmpath` algorithm modifies this slightly by first considering the typical generalized linear model formula

$$\hat{\boldsymbol{\beta}} = \underset{\boldsymbol{\beta}}{\text{argmax}} L(\mathbf{y}; \boldsymbol{\beta})$$

where L denotes the appropriate likelihood function. The `glmpath` algorithm then adds an analagous LASSO penalty term to help with variable selection when $p > n$

$$\hat{\boldsymbol{\beta}}(\lambda) = \underset{\boldsymbol{\beta}}{\text{argmin}}\{-\log L(\mathbf{y}; \boldsymbol{\beta}) + \lambda ||\boldsymbol{\beta}||_1\}$$

where $\lambda > 0$ is the regularization parameter. The `glmpath` algorithm computes coefficient

estimates as λ varies. The algorithm starts with the largest λ that makes $\hat{\boldsymbol{\beta}}(\lambda)$ nonzero with each step using a smaller λ. Each optimization consists of three parts: determining the step size in λ, predicting the corresponding change in the coefficients, and correcting the error in the previous prediction [Park and Hastie, 2007]. The algorithm continues finding the next largest λ that will change the coefficient estimates until no further predictors can be found. However, when the predictors are strongly correlated the coefficient estimates become highly unstable using the L_1 norm penalization [Hastie et al., 2007]. Thus, the `glmpath` algorithm adds a quadratic penalty term and computes the solution to

$$\hat{\boldsymbol{\beta}}(\lambda_1) = \underset{\boldsymbol{\beta}}{\operatorname{argmin}}\{-\log L(\mathbf{y};\boldsymbol{\beta}) + \lambda_1||\boldsymbol{\beta}||_1 + \frac{\lambda_2}{2}||\boldsymbol{\beta}||_2^2\}$$

where $\lambda_1 \in (0,\infty)$ and λ_2 is a fixed, small, positive constant. By adding this quadratic penalty, the effects of the strong correlations do not affect the stability of the fit. Further, when the correlations are not strong, the effects of the quadratic penalty are neglible [Hastie et al., 2007]. Thus, the `glmpath` algorithm uses both the L_1 and L_2 penalties as its default method.

The `glmpath` algorithm uses a default binomial distribution with a logit link and $\lambda_2 =$ .00001. The algorithm also allows for a Poisson distribution with a log link and Gaussian distribution with an identity link. The algorithm then computes the regularization path for generalized linear models with L1 penalty.

4.2.5 Simulations

Simulations are a useful technique to test how well a new methodology performs. In this case, we wished to quantify how accurately the GMIFS method estimated true non-zero coefficients and predicted count data. Furthermore, we wished to determine how the GMIFS method compared to the `glmpath` method in predicting the count outcome and simulations provide a good platform to accomplish this comparison. Several general steps must be considered in the simulation process; how to simulate the response, how to simulate the

predictors associated with the response and how to simulate the predictors not associated with the response. Furthermore, we wished to examine how the methods perform under ideal situations and non-ideal situations such as when distributional assumptions are met and are not met respectively. Note that all simulations were performed using the R programming environment (version 3.1.1) [R Core Team, 2014].

First, we considered the situation where the response is Poisson distributed and the user fits a Poisson regression model. Then we generated the response to follow a Poisson distribution where an offset (a) was and (b) was not used. The uniform distribution was used to generate the predictors. The steps involved in simulating the data under these conditions were as follows:

1. Randomly generate P variables, $x_{i1}, x_{i2}, ..., x_{iP}$ where $i = 1$ to n, using the uniform distribution on the [0,1] interval.

2. Choose P_1 of the P variables to be associated with the response.

3. Assign the P_1 β values associated with the response and the intercept value, α. If the offset is to vary, then a uniform distribution was used with maximum 2200 and minimum 1800 and rounded to the nearest integer. This range was selected because it is recommended to score MN using 2000 cells.

4. Generate the λ values for the Poisson distribution using the following formula:

$$\lambda_i = \exp(\alpha + \log(\text{offset}_i) + \sum_{k=1}^{P_1} \beta_k \mathbf{x}_{ik}).$$

5. Randomly generate $Y_i \sim \text{Poisson}(\lambda_i)$.

6. Fit a Poisson GMIFS model and fit a `glmpath` model.

7. Repeat steps 1-6 r times.

This simulation method was adjusted in several places. In this case we chose $n = 30$ and $n = 80$ which are roughly the sample sizes of our cord blood and breast cancer datasets, respectively. We studied the models letting $P = 100$ predictor variables, $P_1 = 5$ predictor variables associated with the response, and $r = 100$ simulations were used. The intercept (α) and the five predictor variables associated with the response ($\beta_1, \beta_2, \beta_3, \beta_4, \beta_5$) were set to -5, 0.3, 0.2, -0.7, 0.5 and 0.1 respectively for data simulated using no offset. For data simulated using an offset, α was set to -7. This was done to keep lambda values low so the Gaussian approximation for the Poisson distribution is not appropriate. To compare the two different statistical models, the following three outcomes were examined:

A. The number of true predictors that have a non-zero coefficient;

B. The number of false predictors that have a non-zero coefficient;

C. Accuracy of count predictions from the model (sum of squared residuals) when applied to an independent test set.

The methods were compared with and without the use of an offset during the simulation process. Furthermore, the `glmpath` method allows for the use of Gaussian and Poisson distributions. Thus, those options were also used to see what effects user error had on the results. Thus, a total of three models were compared when the true distribution was Poisson:

1. Poisson GMIFS model;

2. `glmpath` using "poisson" family option and lambda2=0 which fits a LASSO model; and

3. `glmpath` using "gaussian" family option and lambda2=0 which fits a LASSO model.

4.3 Results

4.3.1 Simulations

Simulations were performed as described in section 4.2.5 and Figures 20- 22 show the results of the simulations. Figure 20 shows the distribution of the number of predictors correctly identified as non-zero over 100 simulations and the types of models used. The data were generated using both $n = 30$ and $n = 80$ observations. The median number of correctly identified non-zero coefficients with no offset using GMIFS is 1 (range=0, 3) for $n = 30$ and 2 (range=0, 4) for $n = 80$. Similarly, the median number of correctly identified non-zero coefficients with no offset using `glmpath` with Poisson family is 1 (range=0, 5) for $n = 30$ and 2 (range=0, 4) for $n = 80$. This number increases slightly when using the `glmpath` with Gaussian family to a median of 2 (range=0, 5) for $n = 30$ and 4 (range=2, 5) for $n = 80$. All the numbers are similar when an offset is used to generate the data. The median correct using GMIFS is 0 (range=0, 3) for $n = 30$ and 1 (range=0, 4) for $n = 80$. The median correct using `glmpath` with Poisson family is 0 (range=0, 3) for $n = 30$ and 1 (range=0, 4) for $n = 80$. Once again the median values increase when using the `glmpath` with Gaussian family to 2 (range=0, 5) for $n = 30$ and 4 (range=2, 5) for $n = 80$.

Figure 21 shows the distribution of the number of predictors incorrectly identified as non-zero over 100 simulations and the types of models used. The data were generated using both $n = 30$ and $n = 80$ observations. The median number of incorrectly identified non-zero coefficients with no offset using GMIFS is 3 (range=0, 15) for $n = 30$ and 7 (range=0, 28) for $n = 80$. Similarly, the median number of incorrectly identified non-zero coefficients with no offset using `glmpath` with Poisson family is 3 (range=0, 17) for $n = 30$ and 7 (range=0, 41) for $n = 80$. This number increases when using the `glmpath` with Gaussian family to a median of 26 (range=23, 28) for $n = 30$ and 74 (range=73, 76) for $n = 80$. All results are similar when an offset is used to generate the data. The median incorrect using GMIFS is 2 (range=0, 14) for $n = 30$ and 5 (range=0, 26) for $n = 80$. The median incorrect using

`glmpath` with Poisson family is 2 (range=0, 24) for $n = 30$ and 4.5 (range=0, 31) for $n = 80$. Once again the median values increase when using the `glmpath` with Gaussian family to 26 (range=23, 28) for $n = 30$ and 74 (range=72, 76) for $n = 80$.

Figure 22 shows the distribution of the sum of residuals squared as a measure of the model prediction accuracy. The data were generated using both $n = 30$ and $n = 80$ observations. For both sample sizes a learning data set was used to estimate coefficients and then the model was applied to an independent test data set. The median accuracy with no offset using GMIFS is 133 (range=68, 240) for $n = 30$ and 325 (range=188, 699) for $n = 80$. Similarly, the median accuracy with no offset using `glmpath` with Poisson family is 142 (range=55, 254) for $n = 30$ and 333 (range=185, 1666) for $n = 80$. The median accuracy with no offset using `glmpath` with Gaussian family is 206 (range=90, 383) for $n = 30$ and 1,503 (range=535, 3772) for $n = 80$. The numbers are different when an offset is used to generate the data. The median accuracy using GMIFS is 80 (range=30, 185) for $n = 30$ and 205 (range=137, 367) for $n = 80$. The median accuracy using `glmpath` with Poisson family is 80 (range=33, 805) for $n = 30$ and 206 (range=126, 339) for $n = 80$. The median accuracy with an offset using `glmpath` with Gaussian family for both sample sizes is above 50,000.

Figure 20: Number of Predictors Correctly Identified as Non-zero: This figure shows the distribution of the number of predictors correctly identified as non-zero over 100 simulations. There were 5 predictors had non-zero coefficients. Boxplots are separated by the type of distribution used to generate the data and number of observations.

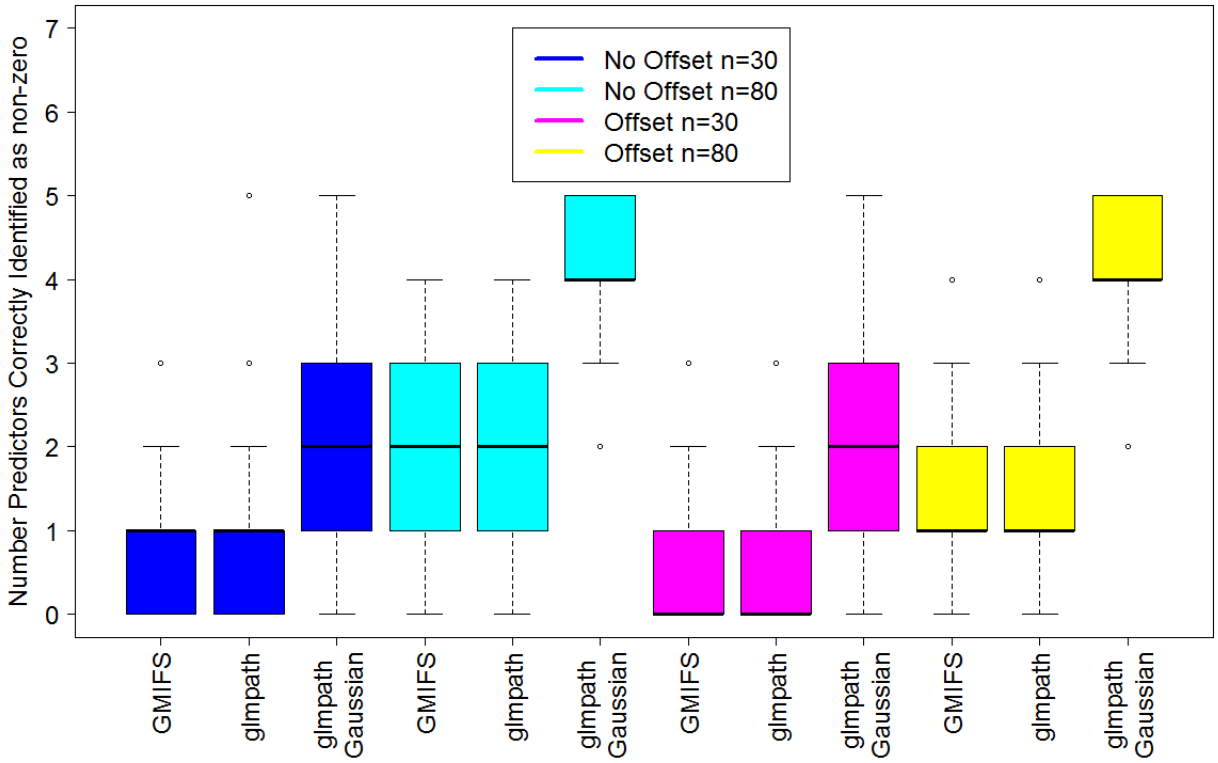


Figure 21: Number of Predictors Incorrectly Identified as Non-zero: This figure shows the distribution of the number of predictors incorrectly identified as non-zero over 100 simulations. There were 95 predictors that had coefficients set to zero. Boxplots are separated by the type of distribution used to generate the data and number of observations.

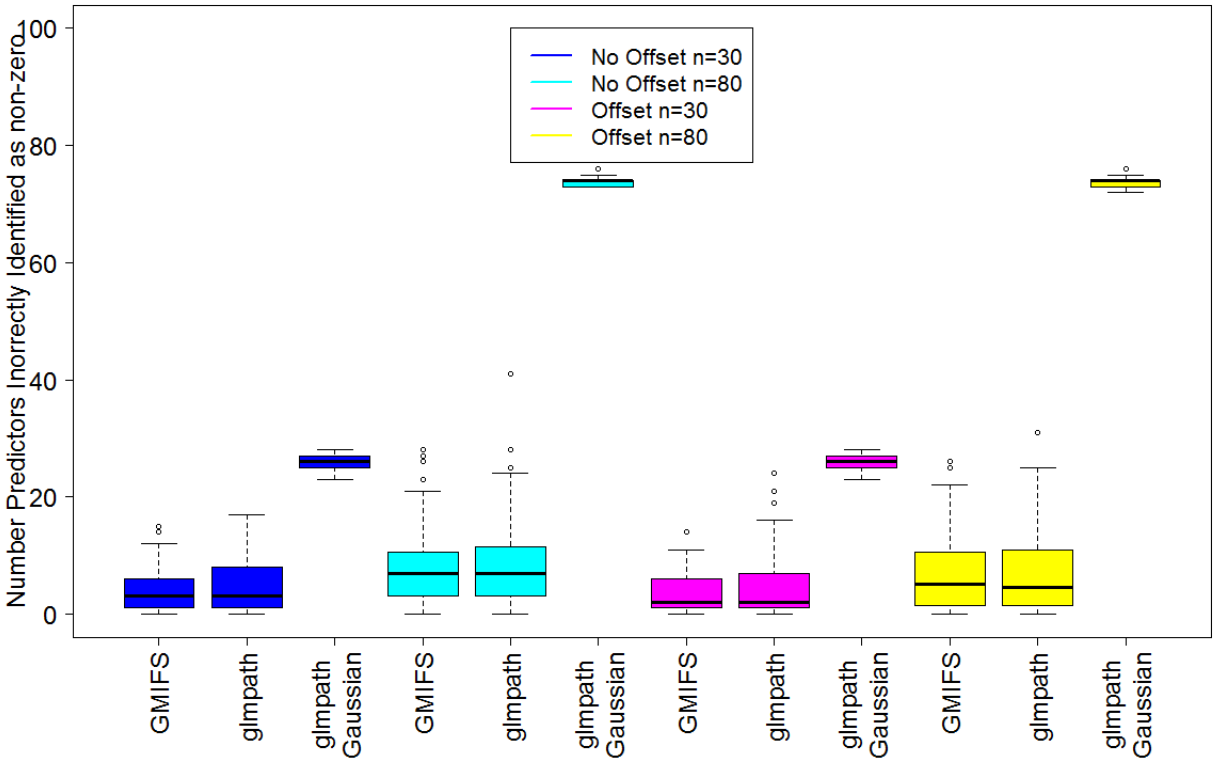
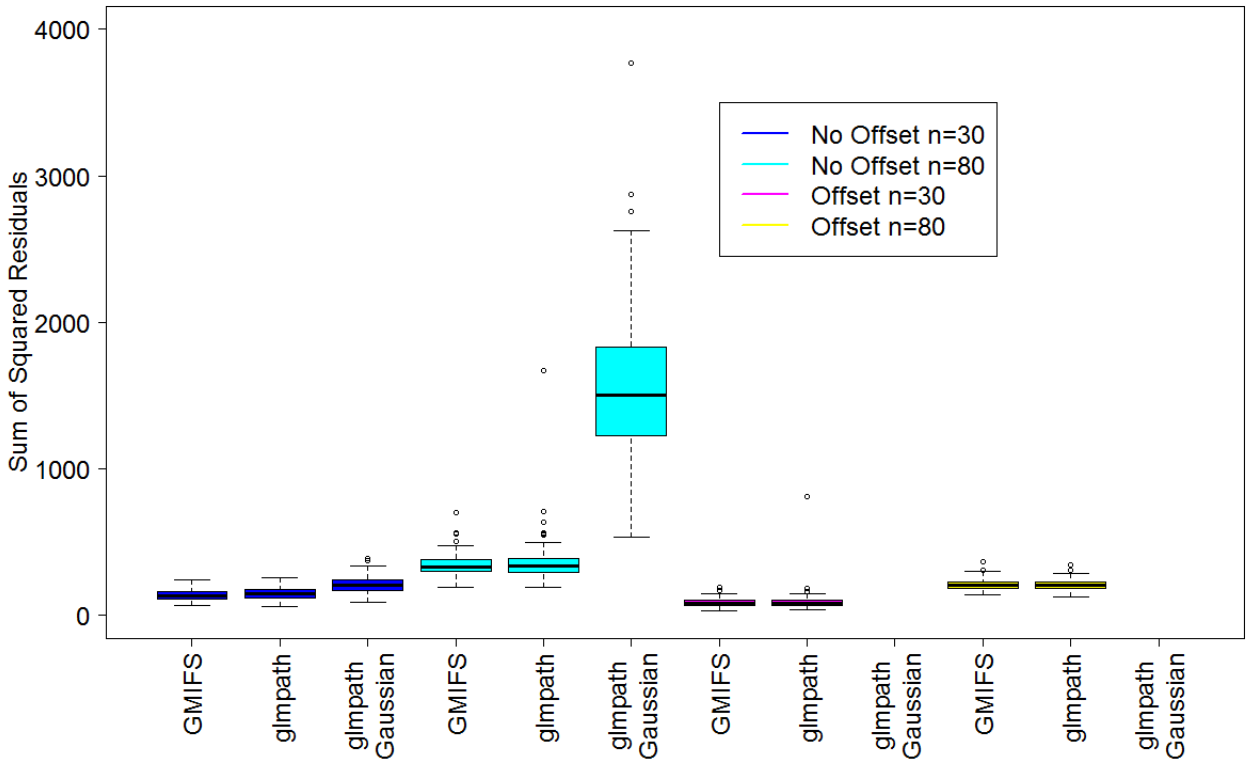


Figure 22: Accuracy of Count Predictions: This figure shows the distribution of the sum of residuals squared over 100 simulations using a learning data set and an independent test data set. Boxplots are separated by the type of distribution used to generate the data and number of observations. The results for `glmpath` with Gaussian family using an offset are not displayed because both values are above 50,000.



4.3.2 Gene Expression Analysis

The cord blood data were collected as part of the Norwegian Mother and Child Cohort Study (Moba) [Magnus et al., 2006]. The target population of Moba is all women who give birth in Norway. The overall goal of this study was to collect data on pregnant women and their children to estimate the association between exposures and diseases. Specifically, the data are part of a subcohort called BraMat, which translates to "good food" in English. This subcohort concentrates on what effect a pregnant woman's diet has on her child. Umbilical cord blood samples were collected immediately after birth from 200 babies. After quality

control and other exclusions, 111 samples were hybridized to Agilent 4x44k human oligonucleotide microarrays to measure gene expression. Of the 111 subjects, 29 also had MN data collected. The MN were scored using the procedure described by Decordier et al. [2009]. Further, demographics such as gender were collected for all subjects. Data were downloaded from Gene Expression Omnibus (GSE31836). Sample processing, image analysis, normalization, background correction and filtering are described in Hochstenbach et al. [2012]. For this analysis the data were further filtered to only include genes that had no missing values, leaving 8497 genes for statistical modeling.

Both GMIFS and `glmpath` models were applied to the cord blood gene expression data set described above. For `glmpath`, the Poisson family option was used and the lambda2 option was set to zero. For GMIFS, the default options were chosen. The response in the model was MN counts and the predictors were the gene expression intensities. Gender was included in the model as part of the unpenalized subset. Based on Figure 23, a Poisson distribution was assumed for both models since the data appear skewed. The final model parameters were chosen using the minimum AIC. The GMIFS model identified 17 non-zero gene expression coefficients as associated with MN count and `glmpath` with Poisson family identified 23. Out of the genes that were identified, 10 were common to both models. Figures 24 (Sum of squared residuals = 101.7) and 25 (Sum of squared residuals = 1.8) show that both models seem to predict MN relatively well. Because simulations showed that `glmpath` seems to overfit, `glmpath`'s ability to predict MN frequency was looked at using only 17 non-zero coefficients . Figure 26 (Sum of squared residuals = 80.0) shows that when the same number of coefficients are used both GMIFS and `glmpath` predict MN frequency with similar accuracy. Table 10 shows the genes that both models identified as being associated with MN count and the types of cancer with which they are linked. Nine out of the ten genes are linked to some type of cancer.

Table 10: Genes Identified as Associated with MN Count by both GMIFS and `glmpath`

Probe ID	Gene Symbol	Gene Name	Associated with Cancer	GMIFS	`glmpath`
A-23-P100196	USP10	ubiquitin specific peptidase 10	Glioblastoma multiforme [Grunda et al., 2006]	X	X
A-23-P138967	SDHD	succinate dehydrogenase complex	Tumor Suppressor [King et al., 2006]	X	X
A-23-P42331	HMGA1	high mobility group AT-hook 1	Pancreatic Adenocarcinoma [Liau et al., 2008]	X	X
A-23-P9293	TJP2	tight junction protein 2	Breast [Martin et al., 2004]	X	X
A-24-P19410	CBX7	chromobox homolog 7	Carcinomas [Federico et al., 2009]	X	X
A-24-P214858	TREML2	triggering receptor expressed on myeloid cells-like 2	Pancreatic [Loos et al., 2009]	X	X
A-24-P2463	WHSC1	Wolf-Hirschhorn syndrome candidate 1	Carcinogenesis [Toyokawa et al., 2011]	X	X
A-24-P397584	TBCC	tubulin folding cofactor C	None Found	X	X
A-24-P398064	KIAA0258	KIAA0258	Colorectal [Sasaki et al., 2008]	X	X
A-32-P18547	C21ORF57	chromosome 21 open reading frame 57	Breast [Smeets et al., 2011]	X	X
A-23-P103824	FAU	Finkel-Biskis-Reilly murine sarcoma virus (FBR-MuSV) ubiquitously expressed	None Found	X	
A-23-P209394	CFLAR	CASP8 and FADD-like apoptosis regulator	Human cancers [Fulda, 2013]	X	
A-23-P79911	PSMF1	proteasome (prosome, macropain) inhibitor subunit 1 (PI31)	Breast Kuznetsova et al. [2006]	X	
A-24-P202567	ITPKC	inositol 1,4,5-trisphosphate 3-kinase C	Cervical [Yang et al., 2012]	X	
A-24-P31235	EIF5A	eukaryotic translation initiation factor 5A	Chronic myeloid leukemia [Balabanov et al., 2007]	X	
A-24-P405054	C1ORF144	chromosome 1 open reading frame 144	Mantle cell lymphoma [Schraders et al., 2008]	X	
A-32-P156549	C1ORF144			X	
A-23-P118313	GABARAPL2	GABA(A) receptor-associated protein-like 2	Lung [Borczuk et al., 2003]		X
A-23-P143817	MYLK	myosin, light polypeptide kinase	Gastric [Chen et al., 2012]		X
A-23-P156809	LOC642880	similar to FKSG62	None Found		X
A-23-P394304	PDZK1IP1	PDZK1 interacting protein 1	Thyroid [Di Maro et al., 2014]		X
A-23-P39665	SLC11A1	solute carrier family 11, member 1	Esophageal [Zaahl et al., 2005]		X
A-23-P67529	KCNN4	potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4	Colorectal [Lai et al., 2011]		X
A-24-P594683	LOC645592	similar to peptidylprolyl isomerase A isoform 1			X
A-24-P708161					X
A-24-P98086	GNA12	guanine nucleotide binding protein (G protein) alpha 12	Oral [Gan et al., 2011]		X
A-32-P10067					X
A-32-P137849					X
A-32-P169754	LOC145221	EST			X
A-32-P208078	MTHFR	5,10-methylenetetrahydrofolate reductase (NADPH)	Breast [Chen et al., 2005]		X

Figure 23: Histogram of MN Counts for cord blood data set.

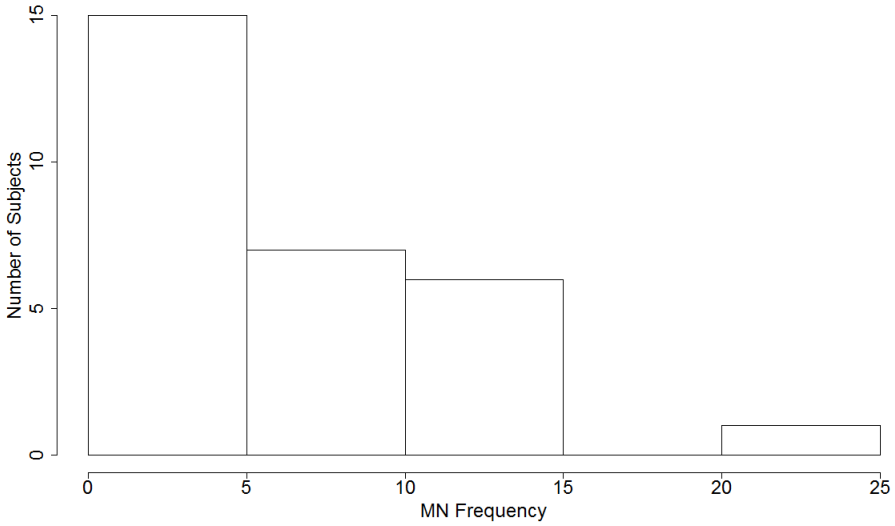


Figure 24: Plot of actual MN counts versus predicted MN counts using GMIFS.

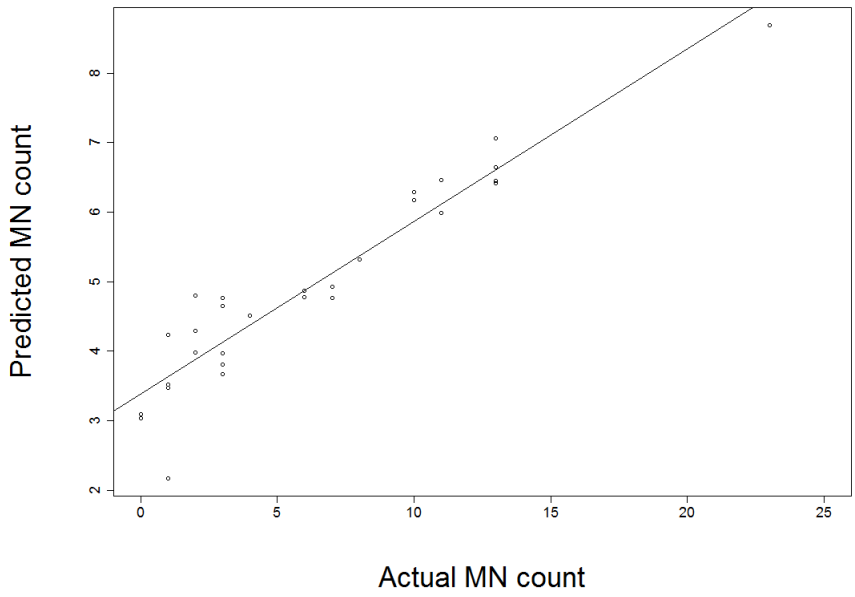


Figure 25: Plot of actual MN counts versus predicted MN counts using `glmpath`.

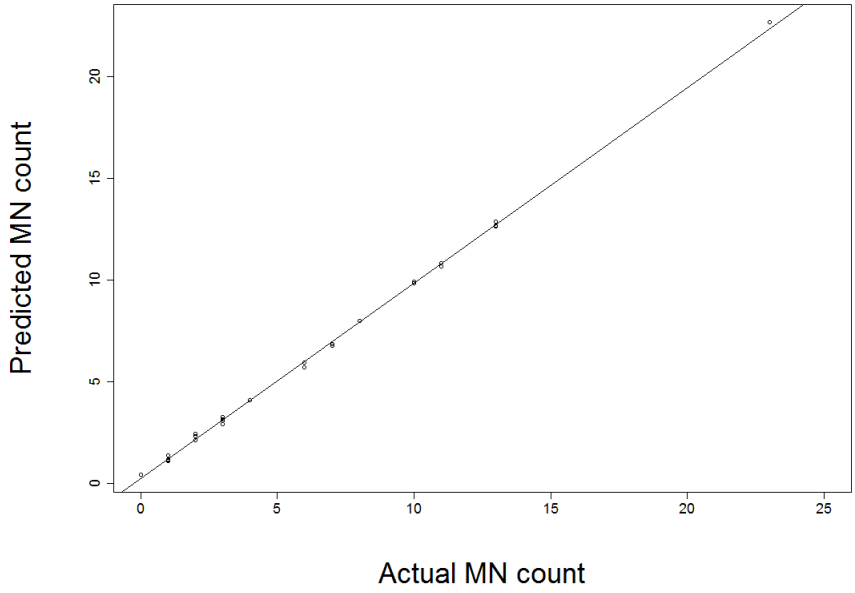
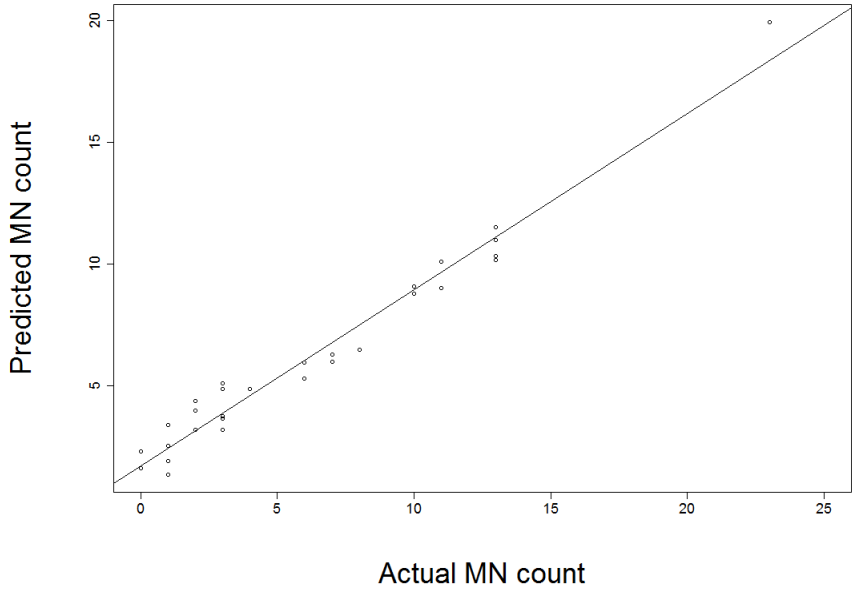


Figure 26: Plot of actual MN counts versus predicted MN counts using `glmpath` using 17 non-zero coefficients.



4.3.3 Methylation Analysis

The breast cancer data were collected as part of an ongoing prospective study titled, Epigenetics and psychoneurologic symptoms in women with breast cancer (R01NR012667) and

includes 70 women diagnosed with breast cancer having both MN frequencies and methylation profiles collected at baseline. MN frequency was typically scored from among 2000 cells with some variation in the total number of cells scored. Specifically, the cells were scored in four sets of 500 cells and mononucleated, binucleated and trinucleated cells were considered in the total. The subjects also all have methylation data available that can be paired with the MN data to test for associations. For each subject MN frequency was calculated as the number of binucleated cells containing one or more nuclei. Further, demographic variables such as age and smoking status were collected for all subjects.

Epigenetics is the "study of heritable changes in gene expression that occur independent of changes in the primary DNA sequence" [Sharma et al., 2010]. The epigenetic gene expression profile is affected by modifications such as DNA methylation, histone modifications, nucleosome positioning and microRNA. Cancer cells undergo drastic changes in the epigenomic landscape compared to normal cells [Sharma et al., 2010]. Alterations in epigenetic mechanisms can lead to silencing of tumor suppresor genes, activation of oncogenes and genetic instability. The first epigenetic alterations identified in cancer were DNA methylation changes. A typical cancer epigenome shows overall global hypomethylation and in contrast hypermethylation of CpG islands [Sharma et al., 2010].

There are several mechanisms that are used to upregulate or downregulate gene expression, one of which is DNA methylation. Essentially this process involves adding a methyl group to a CpG site. A CpG site is a cystine followed by a guanine connected by a phosphate backbone (hence CpG) on a single strand of DNA. There are two important features of CpG sites to consider. The first is rarity; a CpG combination occurs only about one fifth of the expected frequency. The second feature is methylation; CpG sites can be methylated and this accounts for most, if not all, of the methylcytosine in the vertebrate genome [Bird, 1985]. CpG sites can be part of CpG islands, a section of DNA that is rich in CpG sites and are generally present near gene promoters. The methylation status of adjacent CpG sites has been shown to be highly correlated [Bibikova et al., 2011]. This implies that CpG islands are

often methylated or unmethylated as a group. This large scale methylation of CpG islands near promoters has been shown to inhibit transcription of genes. CpG islands are found in approximately 60% of gene promoters [Dedeurwaerder et al., 2011].

Hypomethylation and hypermethylation of specific DNA regions work together to promote tumor growth. Hypomethylation of the genome at repetitive elements, retrotransposons, introns and gene deserts results in the increase of genomic instability. By opening these regions to modification, such as chromosomal rearrangement and transposon translocation, hypomethylation promotes genomic instability which is implicated in many cancers such as thymic lymphomas [Howard et al., 2007]. Similarly, hypomethylation of CpG poor promoters can result in activation of growth promoting genes, such as *R-Ras* and *MAPSIN* in gastric cancer [Sharma et al., 2010]. By activating these types of genes, hypomethylation promotes cancer development and progression. On the other hand, hypermethylation downregulates tumor suppressor genes such as *Rb*, a gene associated with retinoblastoma [Sharma et al., 2010]. Many other genes have been shown to be suppressed by hypermethylation that control functions such as DNA repair, cell cycle, cell adhesion, apoptosis and angiogenesis. All of these mechanisms have been shown to affect cancer development and progression. Thus, studying the epigenomic changes in cancer such as DNA methylation is of great interest. Having a better understanding of these changes can result in possible targets for cancer therapies.

The downregulation of genes with methylated CpG islands can be seen in studies performed on the inactive X chromosome. Females have two X chromosomes and only one needs to be transcriptionally active. Studies have found that many genes on the X chromosome, such as HPRT and G6PD, are made inactive by the methylation of CpG islands [Bird, 1985].

One way to study DNA methylation profiles is to use the Illumina HumanMethylation 450K assay. This assay uses bisulfite conversion followed by hybridization of the sample to an array that interrogates specific CpG sites. Bisulfite treatment of DNA converts unmethylated cytosines into uracils but leaves methylated cytosines unchanged. Since uracil binds to

adenine and cytosine binds to guanine, the bisulfite converted DNA can be differentiated between methylated and unmethylated loci. This can be done by using probes for specific CpG sites and using amplification with marked amino acids to measure whether an adenine or guanine was added. The Illumina 450K methylation assay consists of a chip that contains probes for 482,421 CpG sites [Bibikova et al., 2011]. It is worth mentioning that bisulfite treatment is a harsh process but has been shown to have good reproducibility and precision [Ogino et al., 2006]

The Illumina 450K chip covers 98.9 % of UCSC RefGenes with an average of 17.2 probes per gene. Similarly, the probes target a high proportion of CpG islands. Using in vitro created standards, Bibikova et al. [2011] were able to show that β values (intensity of methylated allele/(intensity of methylated allele + intensity of unmethylated allele + 100)) cluster around the expected value. Bibikova et al. [2011] also tested reproducibility and correlation with whole-genome bisulfite sequencing (WGBS). The 450K assay showed high reproducibility between technical replicates with an R^2 of 0.992. Similarly, there was a high correlation between whole genome bisulfite sequencing (WGBS) and the 450K array. In normal lung cells the correlation was 0.95 and in tumor lung cells the correlation was 0.96 [Bibikova et al., 2011]. This shows that 450K methylation assay provides an accurate and cost-effective method to measure large scale DNA methylation levels.

The numerical summaries per beadtype are β values which can be interpreted as the proportion methylation for that CpG site. Given that there are over 480,000 CpG sites interrogated, it is often necessary to filter the dataset prior to analysis. For example, CpG sites for which all samples have $\beta < 20\%$ are considered completely unmethylated and CpG sites for which all samples have $\beta > 80\%$ are considered completely methylated and both can be filtered from downstream analysis [Zou et al., 2014].

We intended to model MN frequency in the breast cancer data set, however when looking at Figure 27 the MN counts appear to be normally distributed. Thus, we modeled bud frequency, which appear Poisson distributed (Figure 28). Specifically, we were interested in

identifying a multivariable model where methylation levels served as predictor and bud frequency of binucleated cells was the outcome. Buds are similar to MN but do not completely separate from the main nucleus. Buds were calculated in the breast cancer data set as number of cells containing at least one bud. Age and smoking status were included in the model as part of the unpenalized subset. The default options were chosen for GMIFS. The total number of binucleated cells scored was included in the model as an offset. Prior to modeling, methylation sites that were all above 80% methylated and below 20% methylated were filtered out. Further, univariate Poisson models were run on each CpG site where bud frequency was the outcome to further retain only those that were significantly related to buds. CpG sites that had a p-value≤ 0.05 were included in the GMIFS model, leaving 10,860 CpG sites. The final model parameters were chosen using the minimum AIC. The final GMIFS model included 25 non-zero methylation loci coefficients. The model predicts that these 25 loci and subsequently the genes they are associated with Bud formation. Table 11 shows the 25 loci and the genes with which they are associated. Looking at Figure 29, the GMIFS model seems to predict bud frequency relatively well. When `glmpath` was applied to the data, a fatal error occured and the model would not run.

Figure 27: Histogram of MN Counts for breast cancer data set.

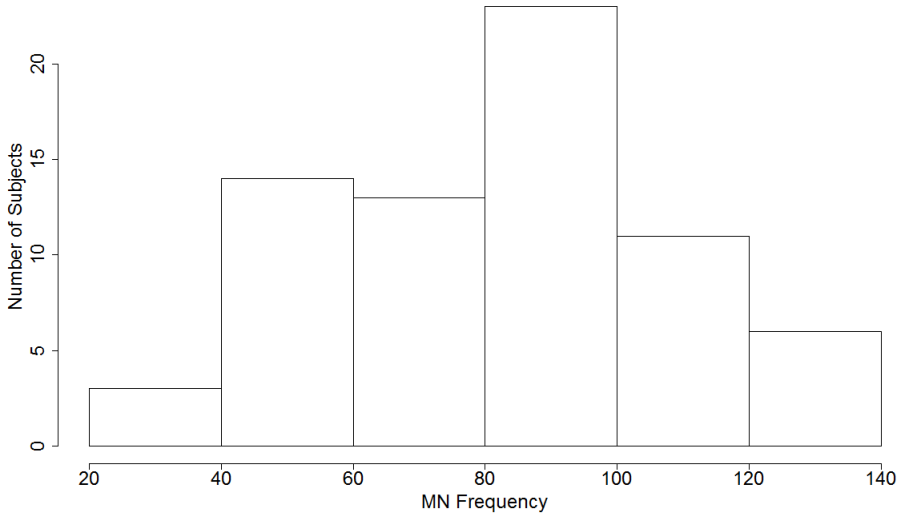


Figure 28: Histogram of bud frquencies in the breast cancer data set.

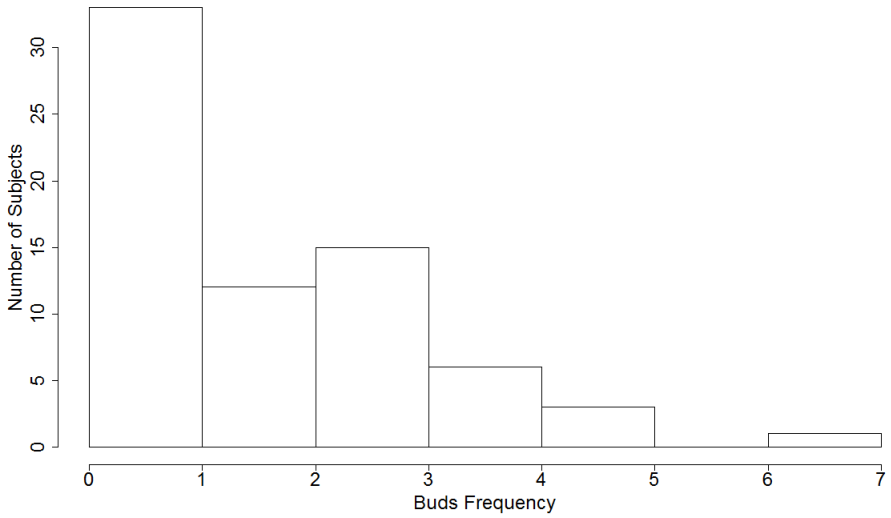


Figure 29: Plot of actual MN counts versus predicted MN counts using GMIFS for breast cancer data.

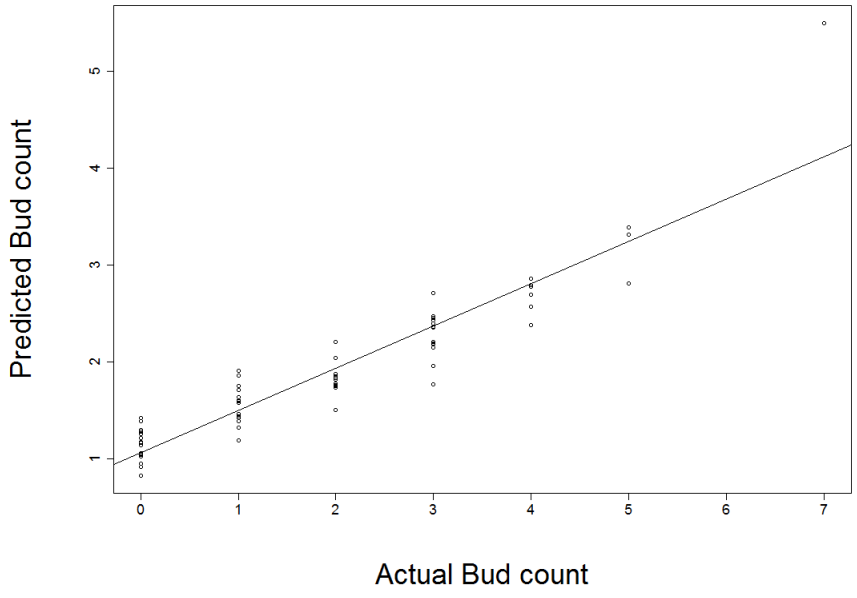


Table 11: Methylation Loci Identified as Associated with Bud Formation

Methylation Loci	Associated Gene	Gene Name	Associated with Cancer
cg21271015			
cg23791325			
cg18598029	HCG27	HLA Complex Group 27	
cg06213635			
cg02402208			
cg06558765	ZFY	Zinc Finger Protein, Y-Linked	
cg21605248			
cg12099459			
cg17594670			
cg13819787	SP9	Sp9 Transcription Factor	
cg26443646			
cg05988603	NCRNA00171	ZNRD1 Antisense RNA 1	Stomach [Hong et al., 2004]
cg10511429			
cg26952662	CTHRC1	Collagen Triple Helix Repeat Containing 1	Solid Cancers [Tang et al., 2006]
cg14140673			
cg15357934	TSPAN31	Tetraspanin 31	Colorectal [Kuhn et al., 2007]
cg15744240	FGF14	Fibroblast Growth Factor 14	Many [Turner and Grose, 2010]
cg12281446	SNAP23	Synaptosomal-Associated Protein	
cg12788108	C17orf64	Chromosome 17 Open Reading Frame 64	Breast [Natrajan et al., 2009]
cg05190096	SHC2	SHC Transforming Protein 2	Breast [Dankort et al., 2001]
cg07054927			
cg21100328	DKFZp434J0226	Uncharacterized LOC93429	
cg23343875	OR10H4	Olfactory Receptor, Family 10, Subfamily H, Member 4	
cg07219314	ZDHHC8P	Zinc Finger, DHHC-Type Containing 8 Pseudogene 1	
cg09692492	ZDHHC8P	Zinc Finger, DHHC-Type Containing 8 Pseudogene 1	

4.4 Discussion

We described the generalized monotone incremental forward stagewise method for modeling a count response when we want to (1) coerce some variables into the model and (2) perform automatic variable selection and model estimation by penalizing predictors. High-throughput data contains more predictors than there are samples so traditional methods are not appropriate in this setting. The GMIFS method was compared to `glmpath`, a popular penalization algorithm. Simulations showed that both methods performed similarly when identifying predictors known to be non-zero. GMIFS appeared to slightly outperform `glmpath` in the sense that GMIFS included fewer predictors that are truly unimportant in the model. This implies `glmpath` tends to overfit. Similarly, when applied to an independent data set GMIFS appeared to have higher predictive accuracy. Also, `glmpath` seems to be unstable and crashed on all attempts to run on our breast cancer data set. Thus, it appears

that GMIFS is more generalizable than `glmpath` to independent data sets.

Both methods were applied to a cord blood gene expression data set. Gene expression profiles were used to predict MN frequency. Both models identified a similar number of genes as related to MN frequency. Further, ten of those genes were common to both models. Nine out of the ten genes have been shown to be associated with different types of cancers. Since MN count is a measure of DNA damage, genes associated with MN frequency would be expected to be linked to cancer. Similarly, both methods were applied breast cancer methylation data set. Both models identified a similar number of methylation sites and 51 of those sites were common to both models.

Both models appear to identify genes linked to cancer. As in the simulations, `glmpath` identified more genes as non-zero compared to GMIFS. In the simulations, this was because `glmpath` was including more predictors incorrectly. However, there is no way to know if this is also the case in the cord blood data set given that these data are observational and no further confirmatory studies can be performed on the samples.

When the models were applied to a breast cancer data set, GMIFS found 25 methylation loci associated with bud formation. Because bud formation is a measure of DNA damage it is a reasonable assumption that these loci are related to genes that are involved in DNA stability. `glmpath`, on the other hand, crashed when the model was run.

5 Conclusions and Future Work

5.1 TCR Analysis Conclusions and Future Work

The first part of this thesis proposed several methods to analyze TCR repertoire data. Specifically, methods to differentiate between patients with and without GVHD after HSCT. The study data consisted of 13 patients that had undergone HSCT. Five patients suffered for GVHD and eight were GVHD-free. Six methods were described and applied that attempted to differentiate the two groups at the VDJ combination level. Three of these methods (CDR3 Distribution Perturbation, Simpson's Diversity Index and Non-parametric Method) calculated different measures for each VDJ combination of the recipients and then permutation tests were used to test for differences. The CDR3 perturbation method calculated using a mean reference and individual reference found three and two significant differences after adjusting for multiple comparisons, respectively. Simpson's diversity index found five significant differences after adjusting for multiple comparisons. The non-parametric method found one significant difference after adjusting for multiple comparisons. The Kullback-Leibler Divergence method was used to compare the difference between CDR3 length distribution at each VDJ combination. This method found almost all VDJ combinations to be statistically different between groups. However, looking at histograms it is difficult to tell what is causing those differences. The proportion logit method used both donor and recipient data to calculate the proportion of shared sequences between donor and recipient at each VDJ combination. Student's T-tests were then used to test for differences. Once again no differences were found between the groups after adjusting for multiple comparison. There were two VDJ combinations that were significant before multiple comparison adjustment. Finally, the Oligoscore method calculated a score for each CDR3 length within a VDJ combination for each group. This method identified several peaks that seem to differ between groups when looked at graphically. These methods were all applied to data collected shortly after the HSCT procedure when the immune systems is not very well reconstituted. Future

work should be done to test for differences in later time points after treatment and prior to treatment. The measures can also be modified to analyze nucleotide or amino acid sequences rather than CDR3 lengths. The HSCT data were also treated as compositional data. No significant differences were found after adjusting for multiple comparisons. All these methods measure different aspects of the T-cell repertoire. Researchers should choose the best measure for the research question they wish to answer. Further, these methodologies should be tried on larger sample sizes to see how they perform in more ideal circumstances.

5.2 Generalized Monotone Incremental Foreward Stagewise Method Conclusions and Future Work

The GMIFS method was extended to analyze count data using the Poisson distribution. Chapter 4 described our GMIFS method and how it performed compared to a popular alternative, `glmpath`. Specifically, analyses related to gene expression and methylation were considered. In both cases, it is important to take into account the cell composition of the sample being tested [Jaffe and Irizarry, 2014]. This is because different cell types have different gene expression and methylation profiles. Houseman et al. [2012] describe a statistical method using methylation data to estimate cell composition. Accounting for varying cell compositions is important because differences between groups could be due to the difference in sample cell composition rather than true biological differences. Not all count data follows a Poisson distribution and GMIFS can be extended to the negative binomial distribution as well. Finally, both the Poisson and negative binomial GMIFS should be extended to the longitudinal setting as well.

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6 R Code

6.1 T-cell Receptor Code

```
1 ###################################################
2 # Reading in the TSV files
3 ###################################################
4
5 #Change file directory to where TSV files are located
6 setwd("C:/Users/makowski/Desktop/Dissertation/TCRdata")
7 #locate TSV files in directory
8 files <- dir(pattern="tsv$")
9 #Function to pull out on sequences marked as productive
10 #and put recipient and donor in one list
11 fileread <- function(name1, name2, file1, file2) {
12 name1<-subset(read.delim(files[file1]),sequenceStatus=="Productive")
13 name2<-subset(read.delim(files[file2]),sequenceStatus=="Productive")
14 list(name1,name2)
15 }
16 #Run the function for each patient
17 pt11<-fileread(name1=ptlld, name2=pt11r3, file1=1, file2=2)
18 pt12<-fileread(name1=ptl2d, name2=pt12r2, file1=3, file2=4)
19 pt13<-fileread(name1=ptl3d, name2=pt13r2, file1=5, file2=6)
20 pt14<-fileread(name1=ptl4d, name2=pt14r3, file1=7, file2=8)
21 pt16<-fileread(name1=ptl6d, name2=pt16r2, file1=9, file2=10)
22 pt24<-fileread(name1=pt24d, name2=pt24r3, file1=11, file2=12)
23 pt3<-fileread(name1=pt3d, name2=pt3r2, file1=13, file2=14)
24 pt4<-fileread(name1=pt4d, name2=pt4r2, file1=15, file2=16)
```

```
pt5<-fileread(name1=pt5d, name2=pt5r2, file1=17, file2=18)
pt6<-fileread(name1=pt6d, name2=pt6r3, file1=19, file2=20)
pt7<-fileread(name1=pt7d, name2=pt7r3, file1=21, file2=22)
pt8<-fileread(name1=pt8d, name2=pt8r3, file1=23, file2=24)
pt9<-fileread(name1=pt9d, name2=pt9r3, file1=25, file2=26)

#function to calculate rpm for both patient and donor
rpm<-function(patient){
norm<-patient[[1]][,6]
depth<-sum(norm)
RPM<-(norm/depth)*1e6
donor<-cbind(patient[[1]],RPM)

norm<-patient[[2]][,6]
depth<-sum(norm)
RPM<-(norm/depth)*1e6
recipient<-cbind(patient[[2]],RPM)

list(donor,recipient)
}

#use function to calculate rpm for donor and recipient
pt11rpm<-rpm(pt11)
rm(pt11)
pt12rpm<-rpm(pt12)
rm(pt12)
pt13rpm<-rpm(pt13)
```

```
rm(pt13)
pt14rpm<-rpm(pt14)
rm(pt14)
pt16rpm<-rpm(pt16)
rm(pt16)
pt24rpm<-rpm(pt24)
rm(pt24)
pt3rpm<-rpm(pt3)
rm(pt3)
pt4rpm<-rpm(pt4)
rm(pt4)
pt5rpm<-rpm(pt5)
rm(pt5)
pt6rpm<-rpm(pt6)
rm(pt6)
pt7rpm<-rpm(pt7)
rm(pt7)
pt8rpm<-rpm(pt8)
rm(pt8)
pt9rpm<-rpm(pt9)
rm(pt9)

#possible V,D and J genes
#Note not all patients have all VDJ combinations interrogated
#by Immunoseq technology so this finds all of them
#I use both Dg and d later and eaier to make both than change more code
```

```
78 J<-unique(c(levels(pt11rpm[[1]]$JGeneName),levels(pt12rpm[[1]]$JGeneName),
      levels(pt13rpm[[1]]$JGeneName),levels(pt14rpm[[1]]$JGeneName),levels(
      pt16rpm[[1]]$JGeneName),levels(pt24rpm[[1]]$JGeneName),levels(pt3rpm
      [[1]]$JGeneName),levels(pt4rpm[[1]]$JGeneName),levels(pt5rpm[[1]]$
      JGeneName),levels(pt6rpm[[1]]$JGeneName),levels(pt7rpm[[1]]$JGeneName),
      levels(pt8rpm[[1]]$JGeneName),levels(pt9rpm[[1]]$JGeneName)))
79 V<-unique(c(levels(pt11rpm[[1]]$VGeneName),levels(pt12rpm[[1]]$VGeneName),
      levels(pt13rpm[[1]]$VGeneName),levels(pt14rpm[[1]]$VGeneName),levels(
      pt16rpm[[1]]$VGeneName),levels(pt24rpm[[1]]$VGeneName),levels(pt3rpm
      [[1]]$VGeneName),levels(pt4rpm[[1]]$VGeneName),levels(pt5rpm[[1]]$
      VGeneName),levels(pt6rpm[[1]]$VGeneName),levels(pt7rpm[[1]]$VGeneName),
      levels(pt8rpm[[1]]$VGeneName),levels(pt9rpm[[1]]$VGeneName)))
80 Dg<-unique(c(levels(pt11rpm[[1]]$DGeneName),levels(pt12rpm[[1]]$DGeneName),
      levels(pt13rpm[[1]]$DGeneName),levels(pt14rpm[[1]]$DGeneName),levels(
      pt16rpm[[1]]$DGeneName),levels(pt24rpm[[1]]$DGeneName),levels(pt3rpm
      [[1]]$DGeneName),levels(pt4rpm[[1]]$DGeneName),levels(pt5rpm[[1]]$
      DGeneName),levels(pt6rpm[[1]]$DGeneName),levels(pt7rpm[[1]]$DGeneName),
      levels(pt8rpm[[1]]$DGeneName),levels(pt9rpm[[1]]$DGeneName)))
81 #remove undefined values
82 V<-V[-1]
83 d<-Dg[-1]
84
85 #save file for later use
86 setwd("C:/Users/makowski/Desktop/Archer Code and Files")
87 save.image("TcellRPM.RData")
88
89
```

```
90 ##############################################
91 #Determine VDJ combinations present in each sample
92 ##############################################
93
94 setwd("C:/Users/makowski/Desktop/Archer Code and Files")
95 load("TcellRPM.RData")
96
97 #Sum all VDJ combinations
98 location<-expand.grid(J,V,d)
99 VDJcomp<-matrix(ncol=1248,nrow=13)
100 for (i in 1:1248){
101 y<-2
102 x<-which(J==location[i,1])
103 w<-which(V==location[i,2])
104 z<-which(d==location[i,3])
105 VDJcomp[,i]<-c(sum(pt11rpm[[y]]$RPM[which(pt11rpm[[y]]$JGeneName==J[x] &
      pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName==d[z])]),
106             sum(pt12rpm[[y]]$RPM[which(pt12rpm[[y]]$JGeneName==J[x] &
                  pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z])
                  ]),
107             sum(pt13rpm[[y]]$RPM[which(pt13rpm[[y]]$JGeneName==J[x] &
                  pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z])
                  ]),
108             sum(pt14rpm[[y]]$RPM[which(pt14rpm[[y]]$JGeneName==J[x] &
                  pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z])
                  ]),
```

```
109             sum(pt16rpm[[y]]$RPM[which(pt16rpm[[y]]$JGeneName==J[x] &
                   pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z])
                   ]),
110             sum(pt24rpm[[y]]$RPM[which(pt24rpm[[y]]$JGeneName==J[x] &
                   pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z])
                   ]),
111             sum(pt3rpm[[y]]$RPM[which(pt3rpm[[y]]$JGeneName==J[x] & pt3rpm
                   [[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z])]),
112             sum(pt4rpm[[y]]$RPM[which(pt4rpm[[y]]$JGeneName==J[x] & pt4rpm
                   [[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z])]),
113             sum(pt5rpm[[y]]$RPM[which(pt5rpm[[y]]$JGeneName==J[x] & pt5rpm
                   [[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z])]),
114             sum(pt6rpm[[y]]$RPM[which(pt6rpm[[y]]$JGeneName==J[x] & pt6rpm
                   [[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z])]),
115             sum(pt7rpm[[y]]$RPM[which(pt7rpm[[y]]$JGeneName==J[x] & pt7rpm
                   [[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z])]),
116             sum(pt8rpm[[y]]$RPM[which(pt8rpm[[y]]$JGeneName==J[x] & pt8rpm
                   [[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z])]),
117             sum(pt9rpm[[y]]$RPM[which(pt9rpm[[y]]$JGeneName==J[x] & pt9rpm
                   [[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z])]))
118             }
119
120 #check to see where 0's are
121 Diversity.check<-matrix(rep(0,(1248*13)),ncol=1248,nrow=13)
122 for (i in 1:13){
123   for (j in 1:1248){
124
```

```
125 if (VDJcomp[i,j]=="0"){
126 Diversity.check[i,j]<-1
127                     }
128    else {
129 Diversity.check[i,j]<-0
130                     }
131              }
132               }
133
134 #keep only VDJ combinations that 7 or more of the sample contain
135 NAs<-c(which(apply(Diversity.check,2,sum)==13),which(apply(Diversity.check
       ,2,sum)==12),which(apply(Diversity.check,2,sum)==11),which(apply(
       Diversity.check,2,sum)==10),which(apply(Diversity.check,2,sum)==9),which
       (apply(Diversity.check,2,sum)==8),which(apply(Diversity.check,2,sum)==7)
       ,which(apply(Diversity.check,2,sum)==6))
136
137 save(NAs,file="VDJNAs.RData")
138
139 ####################################
140 #CDR3 Perturbation Test using mean
141 ####################################
142 #loaded the needed file
143 setwd("C:/Users/makowski/Desktop/Archer Code and Files")
144 load("TcellRPM.RData")
145
146 #Function that does the perturbation test for the VDJ families; x is the J
       gene, w is V gene and z is d gene and y=1 is donor and y=2 is recipient
```

```
147 Perttest<-function(x,w,z,y){
148 #this part calculates the number of unique CDR3 lengths
149 L=length(unique(c(unique(pt11rpm[[y]]$cdr3Length[which(pt11rpm[[y]]$
      JGeneName==J[x] & pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName
      ==d[z])]),
150         unique(pt12rpm[[y]]$cdr3Length[which(pt12rpm[[y]]$JGeneName==J[x] &
              pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z])]),
151         unique(pt13rpm[[y]]$cdr3Length[which(pt13rpm[[y]]$JGeneName==J[x] &
              pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z])]),
152         unique(pt14rpm[[y]]$cdr3Length[which(pt14rpm[[y]]$JGeneName==J[x] &
              pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z])]),
153         unique(pt16rpm[[y]]$cdr3Length[which(pt16rpm[[y]]$JGeneName==J[x]&
             pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z])]),
154         unique(pt24rpm[[y]]$cdr3Length[which(pt24rpm[[y]]$JGeneName==J[x]&
             pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z])]),
155         unique(pt3rpm[[y]]$cdr3Length[which(pt3rpm[[y]]$JGeneName==J[x] &
             pt3rpm[[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z])]),
156         unique(pt4rpm[[y]]$cdr3Length[which(pt4rpm[[y]]$JGeneName==J[x] &
             pt4rpm[[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z])]),
157         unique(pt5rpm[[y]]$cdr3Length[which(pt5rpm[[y]]$JGeneName==J[x] &
             pt5rpm[[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z])]),
158         unique(pt6rpm[[y]]$cdr3Length[which(pt6rpm[[y]]$JGeneName==J[x] &
             pt6rpm[[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z])]),
159         unique(pt7rpm[[y]]$cdr3Length[which(pt7rpm[[y]]$JGeneName==J[x] &
             pt7rpm[[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z])]),
160         unique(pt8rpm[[y]]$cdr3Length[which(pt8rpm[[y]]$JGeneName==J[x] &
             pt8rpm[[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z])]),
```

```
161         unique(pt9rpm[[y]]$cdr3Length[which(pt9rpm[[y]]$JGeneName==J[x] &
                pt9rpm[[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z])])))
162 #print(L)
163
164 #this sets the perturbation statistic to zero if the VDJ combination is not
        present
165 if (L==0){
166 AbsoluteDeviancei<-rep(0,13)
167 AbsoluteDeviancei
168 }
169
170 else {
171 #This list the peak lengths
172 Peaks=unique(c(unique(pt11rpm[[y]]$cdr3Length[which(pt11rpm[[y]]$JGeneName
       ==J[x] & pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName==d[z])])
       ,
173         unique(pt12rpm[[y]]$cdr3Length[which(pt12rpm[[y]]$JGeneName==J[x] &
                pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z])]),
174         unique(pt13rpm[[y]]$cdr3Length[which(pt13rpm[[y]]$JGeneName==J[x] &
                pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z])]),
175         unique(pt14rpm[[y]]$cdr3Length[which(pt14rpm[[y]]$JGeneName==J[x] &
                pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z])]),
176         unique(pt16rpm[[y]]$cdr3Length[which(pt16rpm[[y]]$JGeneName==J[x]&
                pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z])]),
177         unique(pt24rpm[[y]]$cdr3Length[which(pt24rpm[[y]]$JGeneName==J[x]&
                pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z])]),
```

```
178         unique(pt3rpm[[y]]$cdr3Length[which(pt3rpm[[y]]$JGeneName==J[x] &
               pt3rpm[[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z])]),
179         unique(pt4rpm[[y]]$cdr3Length[which(pt4rpm[[y]]$JGeneName==J[x] &
               pt4rpm[[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z])]),
180         unique(pt5rpm[[y]]$cdr3Length[which(pt5rpm[[y]]$JGeneName==J[x] &
               pt5rpm[[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z])]),
181         unique(pt6rpm[[y]]$cdr3Length[which(pt6rpm[[y]]$JGeneName==J[x] &
               pt6rpm[[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z])]),
182         unique(pt7rpm[[y]]$cdr3Length[which(pt7rpm[[y]]$JGeneName==J[x] &
               pt7rpm[[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z])]),
183         unique(pt8rpm[[y]]$cdr3Length[which(pt8rpm[[y]]$JGeneName==J[x] &
               pt8rpm[[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z])]),
184         unique(pt9rpm[[y]]$cdr3Length[which(pt9rpm[[y]]$JGeneName==J[x] &
               pt9rpm[[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z])])))
185 #print(Peaks)
186 #this calculates the total rpm in each peack
187 Peaksi<-matrix(ncol=13,nrow=L)
188 for (i in 1:L){
189 Peaksi[i,]<-c(sum(pt11rpm[[y]]$RPM[which(pt11rpm[[y]]$JGeneName==J[x] &
      pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName==d[z] & pt11rpm[[y
      ]]$cdr3Length==Peaks[i])]),
190             sum(pt12rpm[[y]]$RPM[which(pt12rpm[[y]]$JGeneName==J[x] &
                   pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z]
                   & pt12rpm[[y]]$cdr3Length==Peaks[i])]),
191             sum(pt13rpm[[y]]$RPM[which(pt13rpm[[y]]$JGeneName==J[x] &
                   pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z]
                   & pt13rpm[[y]]$cdr3Length==Peaks[i])]),
```

```
192             sum(pt14rpm[[y]]$RPM[which(pt14rpm[[y]]$JGeneName==J[x] &
                   pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z]
                   & pt14rpm[[y]]$cdr3Length==Peaks[i])]),
193             sum(pt16rpm[[y]]$RPM[which(pt16rpm[[y]]$JGeneName==J[x] &
                   pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z]
                   & pt16rpm[[y]]$cdr3Length==Peaks[i])]),
194             sum(pt24rpm[[y]]$RPM[which(pt24rpm[[y]]$JGeneName==J[x] &
                   pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z]
                   & pt24rpm[[y]]$cdr3Length==Peaks[i])]),
195             sum(pt3rpm[[y]]$RPM[which(pt3rpm[[y]]$JGeneName==J[x] & pt3rpm
                   [[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z] & pt3rpm
                   [[y]]$cdr3Length==Peaks[i])]),
196             sum(pt4rpm[[y]]$RPM[which(pt4rpm[[y]]$JGeneName==J[x] & pt4rpm
                   [[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z] & pt4rpm
                   [[y]]$cdr3Length==Peaks[i])]),
197             sum(pt5rpm[[y]]$RPM[which(pt5rpm[[y]]$JGeneName==J[x] & pt5rpm
                   [[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z] & pt5rpm
                   [[y]]$cdr3Length==Peaks[i])]),
198             sum(pt6rpm[[y]]$RPM[which(pt6rpm[[y]]$JGeneName==J[x] & pt6rpm
                   [[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z] & pt6rpm
                   [[y]]$cdr3Length==Peaks[i])]),
199             sum(pt7rpm[[y]]$RPM[which(pt7rpm[[y]]$JGeneName==J[x] & pt7rpm
                   [[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z] & pt7rpm
                   [[y]]$cdr3Length==Peaks[i])]),
200             sum(pt8rpm[[y]]$RPM[which(pt8rpm[[y]]$JGeneName==J[x] & pt8rpm
                   [[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z] & pt8rpm
                   [[y]]$cdr3Length==Peaks[i])]),
```

```
201             sum(pt9rpm[[y]]$RPM[which(pt9rpm[[y]]$JGeneName==J[x] & pt9rpm
                    [[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z] & pt9rpm
                    [[y]]$cdr3Length==Peaks[i])]))
202             }
203 #print(Peaksi)
204 #total rpm per patient
205 Peakssum<-apply(Peaksi,2,sum)
206
207 #print(Peakssum)
208 #calculates the probability of each peak
209 Probabilityi<-matrix(ncol=13,nrow=L)
210 for (j in 1:L) {
211 Probabilityi[j,]<-Peaksi[j,]/Peakssum
212 }
213
214 #print(Probabilityi)
215 #calculates total rpm in each donor peak
216 controlPeaksi<-matrix(ncol=13,nrow=L)
217 for (k in 1:L){
218 controlPeaksi[k,]<-c(sum(pt11rpm[[1]]$RPM[which(pt11rpm[[1]]$JGeneName==J[x]
       & pt11rpm[[1]]$VGeneName==V[w] & pt11rpm[[1]]$DGeneName==d[z] & pt11rpm
      [[1]]$cdr3Length==Peaks[k])]),
219             sum(pt12rpm[[1]]$RPM[which(pt12rpm[[1]]$JGeneName==J[x] &
                    pt12rpm[[1]]$VGeneName==V[w] & pt12rpm[[1]]$DGeneName==d[z]
                    & pt12rpm[[1]]$cdr3Length==Peaks[k])]),
220             sum(pt13rpm[[1]]$RPM[which(pt13rpm[[1]]$JGeneName==J[x] &
                    pt13rpm[[1]]$VGeneName==V[w] & pt13rpm[[1]]$DGeneName==d[z]
```

```
    & pt13rpm[[1]]$cdr3Length==Peaks[k])]),
221 sum(pt14rpm[[1]]$RPM[which(pt14rpm[[1]]$JGeneName==J[x] &
    pt14rpm[[1]]$VGeneName==V[w] & pt14rpm[[1]]$DGeneName==d[z]
    & pt14rpm[[1]]$cdr3Length==Peaks[k])]),
222 sum(pt16rpm[[1]]$RPM[which(pt16rpm[[1]]$JGeneName==J[x] &
    pt16rpm[[1]]$VGeneName==V[w] & pt16rpm[[1]]$DGeneName==d[z]
    & pt16rpm[[1]]$cdr3Length==Peaks[k])]),
223 sum(pt24rpm[[1]]$RPM[which(pt24rpm[[1]]$JGeneName==J[x] &
    pt24rpm[[1]]$VGeneName==V[w] & pt24rpm[[1]]$DGeneName==d[z]
    & pt24rpm[[1]]$cdr3Length==Peaks[k])]),
224 sum(pt3rpm[[1]]$RPM[which(pt3rpm[[1]]$JGeneName==J[x] & pt3rpm
    [[1]]$VGeneName==V[w] & pt3rpm[[1]]$DGeneName==d[z] & pt3rpm
    [[1]]$cdr3Length==Peaks[k])]),
225 sum(pt4rpm[[1]]$RPM[which(pt4rpm[[1]]$JGeneName==J[x] & pt4rpm
    [[1]]$VGeneName==V[w] & pt4rpm[[1]]$DGeneName==d[z] & pt4rpm
    [[1]]$cdr3Length==Peaks[k])]),
226 sum(pt5rpm[[1]]$RPM[which(pt5rpm[[1]]$JGeneName==J[x] & pt5rpm
    [[1]]$VGeneName==V[w] & pt5rpm[[1]]$DGeneName==d[z] & pt5rpm
    [[1]]$cdr3Length==Peaks[k])]),
227 sum(pt6rpm[[1]]$RPM[which(pt6rpm[[1]]$JGeneName==J[x] & pt6rpm
    [[1]]$VGeneName==V[w] & pt6rpm[[1]]$DGeneName==d[z] & pt6rpm
    [[1]]$cdr3Length==Peaks[k])]),
228 sum(pt7rpm[[1]]$RPM[which(pt7rpm[[1]]$JGeneName==J[x] & pt7rpm
    [[1]]$VGeneName==V[w] & pt7rpm[[1]]$DGeneName==d[z] & pt7rpm
    [[1]]$cdr3Length==Peaks[k])]),
229 sum(pt8rpm[[1]]$RPM[which(pt8rpm[[1]]$JGeneName==J[x] & pt8rpm
    [[1]]$VGeneName==V[w] & pt8rpm[[1]]$DGeneName==d[z] & pt8rpm
```

```
                [[1]]$cdr3Length==Peaks[k])]),
230             sum(pt9rpm[[1]]$RPM[which(pt9rpm[[1]]$JGeneName==J[x] & pt9rpm
                [[1]]$VGeneName==V[w] & pt9rpm[[1]]$DGeneName==d[z] & pt9rpm
                [[1]]$cdr3Length==Peaks[k])]))
231             }
232 #print(controlPeaksi)
233 #calculates total rpm for each donor
234 controlPeakssum<-apply(controlPeaksi,2,sum)
235
236 #print(controlPeakssum)
237 #calculates probability of each donor peak
238 controlProbabilityi<-matrix(ncol=13,nrow=L)
239 for (l in 1:L) {
240 controlProbabilityi[l,]<-controlPeaksi[l,]/controlPeakssum
241 }
242
243 #print(controlProbabilityi)
244 #sets division by zero calculations to zero instead of NaN
245 for (m in 1:L){
246 Probabilityi[m,which(Probabilityi[m,]=="NaN")]<-0
247 controlProbabilityi[m,which(controlProbabilityi[m,]=="NaN")]<-0
248 }
249 #calculates the deviance for mean donor or individual donor
250 Deviancei<-Probabilityi-apply(controlProbabilityi,1,mean)
251 #Change this line to make it a perturbation from each donor instead of mean
252 #Deviancei<-Probabilityi-controlProbabilityi
253 #calculates total deviance per subject
```

```
254 AbsoluteDeviancei<-matrix(nrow=1,ncol=13)
255 for (o in 1:13){
256 AbsoluteDeviancei[o]<-100*sum(abs(Deviancei[,o])/2)
257 }
258 #print(AbsoluteDeviancei)
259
260 AbsoluteDeviancei
261 }
262 }
263 #lists all possible VDJ combinations
264 location<-expand.grid(J,V,d)
265
266 # a is VDJ combination from location object
267 #function calculates perturbation statistic for each VDJ combination
268 AbsoluteDeviance<-matrix(nrow=(length(J)*length(V)*length(d)),ncol=13)
269 for (a in 1:1248){
270 AbsoluteDeviance[a,]<-Perttest(which(J==location[a,1]),which(V==location[a
       ,2]),which(d==location[a,3]),2)
271 print(a)
272 }
273
274 setwd("C:/Users/makowski/Desktop/Archer Code and Files")
275 save(AbsoluteDeviance,location,file="Perturbationtestmean.RData")
276
277
278
279
```

```
280 ##############################################
281 #Run the permutation tests for perturbation test using mean
282 ##############################################
283
284 #set the two groups
285 GroupGVT<-c(2,5,7,8,11)
286 GroupGVHD<-c(1,3,4,6,9,10,12,13)
287 #GVT is 0 and GVHD is 1
288 Group<-c(1,0,1,1,0,1,0,0,1,1,0,1,1)
289
290 #for referencing which VDJ combination
291 rownames(AbsoluteDeviance)<-c(1:1248)
292
293 #load NA values for tests
294 setwd("C:/Users/makowski/Desktop/Archer Code and Files")
295 load("VDJNAs.RData")
296
297 AbsoluteDeviance.NA<-AbsoluteDeviance[-NAs,]
298 #run the permutation test
299 set.seed(123)
300 B <- 1000
301 test.stat<-pval<-numeric()
302 test.stat.b<-matrix(nrow=dim(AbsoluteDeviance.NA)[1], ncol=B)
303 for (i in 1:dim(AbsoluteDeviance.NA)[1]) {
304
305        test.stat[i]<-mean(AbsoluteDeviance.NA[i,Group==1]) - mean(
               AbsoluteDeviance.NA[i,Group==0])
```

```
306         for (j in 1:B) {
307
308                sample<-sample(Group, replace=FALSE)
309                 test.stat.b[i,j]<-mean(AbsoluteDeviance.NA[i,sample==1]) -
                       mean(AbsoluteDeviance.NA[i,sample==0])
310         }
311         pval[i]<-sum(abs(test.stat.b[i,]>abs(test.stat[i])))/B
312 }
313
314 fdr<-cbind(p.adjust(pval, method="BH"),c(1:dim(AbsoluteDeviance.NA)[1]))
315 sum(fdr<0.05)
316 fdr.order<-fdr[order(pval),]
317 location.NA<-location[-NAs,]
318 groupnames<-factor(Group,levels=c(0,1),labels=c("No GVHD","GVHD"))
319 #make some plots
320
321 png(file = "perttestmean.png", width = 1200, height = 1000, units = "px")
322 par(mfrow=c(2,2),mar=c(5.1,4.5,4.1,2.1))
323 plot(groupnames,AbsoluteDeviance.NA[fdr.order[1,2],],main=paste(location.NA[
       fdr.order[1,2],1],location.NA[fdr.order[1,2],2],location.NA[fdr.order
       [1,2],3]),cex.lab=2,cex.main=2,cex.axis=2,ylab="Perturbation")
324 plot(groupnames,AbsoluteDeviance.NA[fdr.order[2,2],],main=paste(location.NA[
       fdr.order[2,2],1],location.NA[fdr.order[2,2],2],location.NA[fdr.order
       [2,2],3]),cex.lab=2,cex.main=2,cex.axis=2,ylab="Perturbation")
325 plot(groupnames,AbsoluteDeviance.NA[fdr.order[3,2],],main=paste(location.NA[
       fdr.order[3,2],1],location.NA[fdr.order[3,2],2],location.NA[fdr.order
       [3,2],3]),cex.lab=2,cex.main=2,cex.axis=2,ylab="Perturbation")
```

```
dev.off()


library(xtable)
apply(t(AbsoluteDeviance[fdr.order[c(1:4),2],GroupGVT]),2,FUN=median)
perttest.table<-cbind(location.NA[fdr.order[c(1:3),2],],apply(t(
    AbsoluteDeviance.NA[fdr.order[c(1:3),2],GroupGVT]),2,FUN=median),apply(t
    (AbsoluteDeviance.NA[fdr.order[c(1:3),2],GroupGVHD]),2,FUN=median),fdr.
    order[c(1:3),1])
colnames(perttest.table)<-c("JGene","VGene","DGene", "Median No GVHD
    Perturbation", "Median GVHD Perturbation", "FDR")
xtable(perttest.table,digits=3)



##################################################
#CDR3 Perturbation Test using individual control
##################################################
#loaded the needed file
setwd("C:/Users/makowski/Desktop/Archer Code and Files")
load("TcellRPM.RData")

#Function that does the perturbation test for the VDJ families; x is the J
    gene, w is V gene and z is d gene and y=1 is donor and y=2 is recipient
Perttest<-function(x,w,z,y){
```

```
#this part calculates the number of unique CDR3 lengths
L=length(unique(c(unique(pt11rpm[[y]]$cdr3Length[which(pt11rpm[[y]]$
    JGeneName==J[x] & pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName
    ==d[z])]),
        unique(pt12rpm[[y]]$cdr3Length[which(pt12rpm[[y]]$JGeneName==J[x] &
            pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z])]),
        unique(pt13rpm[[y]]$cdr3Length[which(pt13rpm[[y]]$JGeneName==J[x] &
            pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z])]),
        unique(pt14rpm[[y]]$cdr3Length[which(pt14rpm[[y]]$JGeneName==J[x] &
            pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z])]),
        unique(pt16rpm[[y]]$cdr3Length[which(pt16rpm[[y]]$JGeneName==J[x]&
           pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z])]),
        unique(pt24rpm[[y]]$cdr3Length[which(pt24rpm[[y]]$JGeneName==J[x]&
           pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z])]),
        unique(pt3rpm[[y]]$cdr3Length[which(pt3rpm[[y]]$JGeneName==J[x] &
           pt3rpm[[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z])]),
        unique(pt4rpm[[y]]$cdr3Length[which(pt4rpm[[y]]$JGeneName==J[x] &
           pt4rpm[[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z])]),
        unique(pt5rpm[[y]]$cdr3Length[which(pt5rpm[[y]]$JGeneName==J[x] &
           pt5rpm[[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z])]),
        unique(pt6rpm[[y]]$cdr3Length[which(pt6rpm[[y]]$JGeneName==J[x] &
           pt6rpm[[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z])]),
        unique(pt7rpm[[y]]$cdr3Length[which(pt7rpm[[y]]$JGeneName==J[x] &
           pt7rpm[[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z])]),
        unique(pt8rpm[[y]]$cdr3Length[which(pt8rpm[[y]]$JGeneName==J[x] &
           pt8rpm[[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z])]),
```

```
361         unique(pt9rpm[[y]]$cdr3Length[which(pt9rpm[[y]]$JGeneName==J[x] &
                pt9rpm[[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z])])))
362 #print(L)
363
364 #this sets the perturbation statistic to zero if the VDJ combination is not
        present
365 if (L==0){
366 AbsoluteDeviancei<-rep(0,13)
367 AbsoluteDeviancei
368 }
369
370 else {
371 #This list the peak lengths
372 Peaks=unique(c(unique(pt11rpm[[y]]$cdr3Length[which(pt11rpm[[y]]$JGeneName
       ==J[x] & pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName==d[z])])
       ,
373         unique(pt12rpm[[y]]$cdr3Length[which(pt12rpm[[y]]$JGeneName==J[x] &
                pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z])]),
374         unique(pt13rpm[[y]]$cdr3Length[which(pt13rpm[[y]]$JGeneName==J[x] &
                pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z])]),
375         unique(pt14rpm[[y]]$cdr3Length[which(pt14rpm[[y]]$JGeneName==J[x] &
                pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z])]),
376         unique(pt16rpm[[y]]$cdr3Length[which(pt16rpm[[y]]$JGeneName==J[x]&
                pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z])]),
377         unique(pt24rpm[[y]]$cdr3Length[which(pt24rpm[[y]]$JGeneName==J[x]&
                pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z])]),
```

```
378         unique(pt3rpm[[y]]$cdr3Length[which(pt3rpm[[y]]$JGeneName==J[x] &
               pt3rpm[[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z])]),
379         unique(pt4rpm[[y]]$cdr3Length[which(pt4rpm[[y]]$JGeneName==J[x] &
               pt4rpm[[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z])]),
380         unique(pt5rpm[[y]]$cdr3Length[which(pt5rpm[[y]]$JGeneName==J[x] &
               pt5rpm[[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z])]),
381         unique(pt6rpm[[y]]$cdr3Length[which(pt6rpm[[y]]$JGeneName==J[x] &
               pt6rpm[[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z])]),
382         unique(pt7rpm[[y]]$cdr3Length[which(pt7rpm[[y]]$JGeneName==J[x] &
               pt7rpm[[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z])]),
383         unique(pt8rpm[[y]]$cdr3Length[which(pt8rpm[[y]]$JGeneName==J[x] &
               pt8rpm[[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z])]),
384         unique(pt9rpm[[y]]$cdr3Length[which(pt9rpm[[y]]$JGeneName==J[x] &
               pt9rpm[[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z])])))
385 #print(Peaks)
386 #this calculates the total rpm in each peack
387 Peaksi<-matrix(ncol=13,nrow=L)
388 for (i in 1:L){
389 Peaksi[i,]<-c(sum(pt11rpm[[y]]$RPM[which(pt11rpm[[y]]$JGeneName==J[x] &
      pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName==d[z] & pt11rpm[[y
      ]]$cdr3Length==Peaks[i])]),
390             sum(pt12rpm[[y]]$RPM[which(pt12rpm[[y]]$JGeneName==J[x] &
                   pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z]
                   & pt12rpm[[y]]$cdr3Length==Peaks[i])]),
391             sum(pt13rpm[[y]]$RPM[which(pt13rpm[[y]]$JGeneName==J[x] &
                   pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z]
                   & pt13rpm[[y]]$cdr3Length==Peaks[i])]),
```

```
392             sum(pt14rpm[[y]]$RPM[which(pt14rpm[[y]]$JGeneName==J[x] &
                   pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z]
                   & pt14rpm[[y]]$cdr3Length==Peaks[i])]),
393             sum(pt16rpm[[y]]$RPM[which(pt16rpm[[y]]$JGeneName==J[x] &
                   pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z]
                   & pt16rpm[[y]]$cdr3Length==Peaks[i])]),
394             sum(pt24rpm[[y]]$RPM[which(pt24rpm[[y]]$JGeneName==J[x] &
                   pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z]
                   & pt24rpm[[y]]$cdr3Length==Peaks[i])]),
395             sum(pt3rpm[[y]]$RPM[which(pt3rpm[[y]]$JGeneName==J[x] & pt3rpm
                   [[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z] & pt3rpm
                   [[y]]$cdr3Length==Peaks[i])]),
396             sum(pt4rpm[[y]]$RPM[which(pt4rpm[[y]]$JGeneName==J[x] & pt4rpm
                   [[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z] & pt4rpm
                   [[y]]$cdr3Length==Peaks[i])]),
397             sum(pt5rpm[[y]]$RPM[which(pt5rpm[[y]]$JGeneName==J[x] & pt5rpm
                   [[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z] & pt5rpm
                   [[y]]$cdr3Length==Peaks[i])]),
398             sum(pt6rpm[[y]]$RPM[which(pt6rpm[[y]]$JGeneName==J[x] & pt6rpm
                   [[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z] & pt6rpm
                   [[y]]$cdr3Length==Peaks[i])]),
399             sum(pt7rpm[[y]]$RPM[which(pt7rpm[[y]]$JGeneName==J[x] & pt7rpm
                   [[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z] & pt7rpm
                   [[y]]$cdr3Length==Peaks[i])]),
400             sum(pt8rpm[[y]]$RPM[which(pt8rpm[[y]]$JGeneName==J[x] & pt8rpm
                   [[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z] & pt8rpm
                   [[y]]$cdr3Length==Peaks[i])]),
```

```
401             sum(pt9rpm[[y]]$RPM[which(pt9rpm[[y]]$JGeneName==J[x] & pt9rpm
                    [[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z] & pt9rpm
                    [[y]]$cdr3Length==Peaks[i])]))
402             }
403 #print(Peaksi)
404 #total rpm per patient
405 Peakssum<-apply(Peaksi,2,sum)
406
407 #print(Peakssum)
408 #calculates the probability of each peak
409 Probabilityi<-matrix(ncol=13,nrow=L)
410 for (j in 1:L) {
411 Probabilityi[j,]<-Peaksi[j,]/Peakssum
412 }
413
414 #print(Probabilityi)
415 #calculates total rpm in each donor peak
416 controlPeaksi<-matrix(ncol=13,nrow=L)
417 for (k in 1:L){
418 controlPeaksi[k,]<-c(sum(pt11rpm[[1]]$RPM[which(pt11rpm[[1]]$JGeneName==J[x]
       & pt11rpm[[1]]$VGeneName==V[w] & pt11rpm[[1]]$DGeneName==d[z] & pt11rpm
      [[1]]$cdr3Length==Peaks[k])]),
419             sum(pt12rpm[[1]]$RPM[which(pt12rpm[[1]]$JGeneName==J[x] &
                    pt12rpm[[1]]$VGeneName==V[w] & pt12rpm[[1]]$DGeneName==d[z]
                    & pt12rpm[[1]]$cdr3Length==Peaks[k])]),
420             sum(pt13rpm[[1]]$RPM[which(pt13rpm[[1]]$JGeneName==J[x] &
                    pt13rpm[[1]]$VGeneName==V[w] & pt13rpm[[1]]$DGeneName==d[z]
```

```
    & pt13rpm[[1]]$cdr3Length==Peaks[k])]),
421         sum(pt14rpm[[1]]$RPM[which(pt14rpm[[1]]$JGeneName==J[x] &
    pt14rpm[[1]]$VGeneName==V[w] & pt14rpm[[1]]$DGeneName==d[z]
    & pt14rpm[[1]]$cdr3Length==Peaks[k])]),
422         sum(pt16rpm[[1]]$RPM[which(pt16rpm[[1]]$JGeneName==J[x] &
    pt16rpm[[1]]$VGeneName==V[w] & pt16rpm[[1]]$DGeneName==d[z]
    & pt16rpm[[1]]$cdr3Length==Peaks[k])]),
423         sum(pt24rpm[[1]]$RPM[which(pt24rpm[[1]]$JGeneName==J[x] &
    pt24rpm[[1]]$VGeneName==V[w] & pt24rpm[[1]]$DGeneName==d[z]
    & pt24rpm[[1]]$cdr3Length==Peaks[k])]),
424         sum(pt3rpm[[1]]$RPM[which(pt3rpm[[1]]$JGeneName==J[x] & pt3rpm
    [[1]]$VGeneName==V[w] & pt3rpm[[1]]$DGeneName==d[z] & pt3rpm
    [[1]]$cdr3Length==Peaks[k])]),
425         sum(pt4rpm[[1]]$RPM[which(pt4rpm[[1]]$JGeneName==J[x] & pt4rpm
    [[1]]$VGeneName==V[w] & pt4rpm[[1]]$DGeneName==d[z] & pt4rpm
    [[1]]$cdr3Length==Peaks[k])]),
426         sum(pt5rpm[[1]]$RPM[which(pt5rpm[[1]]$JGeneName==J[x] & pt5rpm
    [[1]]$VGeneName==V[w] & pt5rpm[[1]]$DGeneName==d[z] & pt5rpm
    [[1]]$cdr3Length==Peaks[k])]),
427         sum(pt6rpm[[1]]$RPM[which(pt6rpm[[1]]$JGeneName==J[x] & pt6rpm
    [[1]]$VGeneName==V[w] & pt6rpm[[1]]$DGeneName==d[z] & pt6rpm
    [[1]]$cdr3Length==Peaks[k])]),
428         sum(pt7rpm[[1]]$RPM[which(pt7rpm[[1]]$JGeneName==J[x] & pt7rpm
    [[1]]$VGeneName==V[w] & pt7rpm[[1]]$DGeneName==d[z] & pt7rpm
    [[1]]$cdr3Length==Peaks[k])]),
429         sum(pt8rpm[[1]]$RPM[which(pt8rpm[[1]]$JGeneName==J[x] & pt8rpm
    [[1]]$VGeneName==V[w] & pt8rpm[[1]]$DGeneName==d[z] & pt8rpm
```

```
                [[1]]$cdr3Length==Peaks[k])]),
430             sum(pt9rpm[[1]]$RPM[which(pt9rpm[[1]]$JGeneName==J[x] & pt9rpm
                [[1]]$VGeneName==V[w] & pt9rpm[[1]]$DGeneName==d[z] & pt9rpm
                [[1]]$cdr3Length==Peaks[k])]))
431             }
432 #print(controlPeaksi)
433 #calculates total rpm for each donor
434 controlPeakssum<-apply(controlPeaksi,2,sum)
435
436 #print(controlPeakssum)
437 #calculates probability of each donor peak
438 controlProbabilityi<-matrix(ncol=13,nrow=L)
439 for (l in 1:L) {
440 controlProbabilityi[l,]<-controlPeaksi[l,]/controlPeakssum
441 }
442
443 #print(controlProbabilityi)
444 #sets division by zero calculations to zero instead of NaN
445 for (m in 1:L){
446 Probabilityi[m,which(Probabilityi[m,]=="NaN")]<-0
447 controlProbabilityi[m,which(controlProbabilityi[m,]=="NaN")]<-0
448 }
449 #calculates the deviance for mean donor or individual donor
450 #Deviancei<-Probabilityi-apply(controlProbabilityi,1,mean)
451 #Change this line to make it a perturbation from each donor instead of mean
452 Deviancei<-Probabilityi-controlProbabilityi
453 #calculates total deviance per subject
```

```
AbsoluteDeviancei<-matrix(nrow=1,ncol=13)
for (o in 1:13){
AbsoluteDeviancei[o]<-100*sum(abs(Deviancei[,o])/2)
}
#print(AbsoluteDeviancei)

AbsoluteDeviancei
}
}
#lists all possible VDJ combinations
location<-expand.grid(J,V,d)

# a is VDJ combination from location object
#function calculates perturbation statistic for each VDJ combination
AbsoluteDeviance<-matrix(nrow=(length(J)*length(V)*length(d)),ncol=13)
for (a in 1:1248){
AbsoluteDeviance[a,]<-Perttest(which(J==location[a,1]),which(V==location[a
    ,2]),which(d==location[a,3]),2)
print(a)
}

setwd("C:/Users/makowski/Desktop/Archer Code and Files")
save(AbsoluteDeviance,location,file="Perturbationtestdonor.RData")
```

```
480 ##############################################
481 #Run the permutation tests for perturbation test using individual control
482 ##############################################
483
484
485 #set the two groups
486 GroupGVT<-c(2,5,7,8,11)
487 GroupGVHD<-c(1,3,4,6,9,10,12,13)
488 #GVT is 0 and GVHD is 1
489 Group<-c(1,0,1,1,0,1,0,0,1,1,0,1,1)
490
491 #for referencing which VDJ combination
492 rownames(AbsoluteDeviance)<-c(1:1248)
493 #load NA values for tests
494 setwd("C:/Users/makowski/Desktop/Archer Code and Files")
495 load("VDJNAs.RData")
496
497 AbsoluteDeviance.NA<-AbsoluteDeviance[-NAs,]
498 #run the permutation test
499 set.seed(123)
500 B <- 1000
501 test.stat<-pval<-numeric()
502 test.stat.b<-matrix(nrow=dim(AbsoluteDeviance.NA)[1], ncol=B)
503 for (i in 1:dim(AbsoluteDeviance.NA)[1]) {
504
505         test.stat[i]<-mean(AbsoluteDeviance.NA[i,Group==1]) - mean(
              AbsoluteDeviance.NA[i,Group==0])
```

```
506         for (j in 1:B) {
507
508                sample<-sample(Group, replace=FALSE)
509                 test.stat.b[i,j]<-mean(AbsoluteDeviance.NA[i,sample==1]) -
                       mean(AbsoluteDeviance.NA[i,sample==0])
510         }
511         pval[i]<-sum(abs(test.stat.b[i,]>abs(test.stat[i])))/B
512 }
513
514 fdr<-cbind(p.adjust(pval, method="BH"),c(1:dim(AbsoluteDeviance.NA)[1]))
515 sum(fdr<0.05)
516 fdr.order<-fdr[order(pval),]
517 location.NA<-location[-NAs,]
518 groupnames<-factor(Group,levels=c(0,1),labels=c("No GVHD","GVHD"))
519 #make some plots
520
521 png(file = "perttestindiv.png", width = 1200, height = 1000, units = "px")
522 par(mfrow=c(1,2),mar=c(5.1,4.5,4.1,2.1))
523 plot(groupnames,AbsoluteDeviance.NA[fdr.order[1,2],],main=paste(location.NA[
       fdr.order[1,2],1],location.NA[fdr.order[1,2],2],location.NA[fdr.order
       [1,2],3]),cex.lab=2,cex.main=2,cex.axis=2,ylab="Perturbation")
524 plot(groupnames,AbsoluteDeviance.NA[fdr.order[2,2],],main=paste(location.NA[
       fdr.order[2,2],1],location.NA[fdr.order[2,2],2],location.NA[fdr.order
       [2,2],3]),cex.lab=2,cex.main=2,cex.axis=2,ylab="Perturbation")
525 dev.off()
526
527
```

```
library(xtable)
apply(t(AbsoluteDeviance.NA[fdr.order[c(1:2),2],GroupGVT]),2,FUN=median)
perttest.table<-cbind(location.NA[fdr.order[c(1:2),2],],apply(t(
    AbsoluteDeviance.NA[fdr.order[c(1:2),2],GroupGVT]),2,FUN=median),apply(t
    (AbsoluteDeviance.NA[fdr.order[c(1:2),2],GroupGVHD]),2,FUN=median),fdr.
    order[c(1:2),1])
colnames(perttest.table)<-c("JGene","VGene","DGene", "Median No GVHD
    Perturbation", "Median GVHD Perturbation", "FDR")
xtable(perttest.table,digits=3)


##########################################
#Calculation of Oligoscores
##########################################

#loaded the needed file
setwd("C:/Users/makowski/Desktop/Archer Code and Files")
load("TcellRPM.RData")

GroupGVT<-c(2,5,7,8,11)
GroupGVHD<-c(1,3,4,6,9,10,12,13)

#Function that does the oliogoscore test for the VDJ families x is the J
    gene, w is V gene and z is d gene and y=1 is donor and y=2 is recipient
oligotest<-function(x,w,z,y){
#this part calculates the number of unique CDR3 lengths
```

```
549 L=length(unique(c(unique(pt11rpm[[y]]$cdr3Length[which(pt11rpm[[y]]$
      JGeneName==J[x] & pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName
      ==d[z])]),
550         unique(pt12rpm[[y]]$cdr3Length[which(pt12rpm[[y]]$JGeneName==J[x] &
                pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z])]),
551         unique(pt13rpm[[y]]$cdr3Length[which(pt13rpm[[y]]$JGeneName==J[x] &
                pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z])]),
552         unique(pt14rpm[[y]]$cdr3Length[which(pt14rpm[[y]]$JGeneName==J[x] &
                pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z])]),
553         unique(pt16rpm[[y]]$cdr3Length[which(pt16rpm[[y]]$JGeneName==J[x]&
                pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z])]),
554         unique(pt24rpm[[y]]$cdr3Length[which(pt24rpm[[y]]$JGeneName==J[x]&
                pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z])]),
555         unique(pt3rpm[[y]]$cdr3Length[which(pt3rpm[[y]]$JGeneName==J[x] &
                pt3rpm[[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z])]),
556         unique(pt4rpm[[y]]$cdr3Length[which(pt4rpm[[y]]$JGeneName==J[x] &
                pt4rpm[[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z])]),
557         unique(pt5rpm[[y]]$cdr3Length[which(pt5rpm[[y]]$JGeneName==J[x] &
                pt5rpm[[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z])]),
558         unique(pt6rpm[[y]]$cdr3Length[which(pt6rpm[[y]]$JGeneName==J[x] &
                pt6rpm[[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z])]),
559         unique(pt7rpm[[y]]$cdr3Length[which(pt7rpm[[y]]$JGeneName==J[x] &
                pt7rpm[[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z])]),
560         unique(pt8rpm[[y]]$cdr3Length[which(pt8rpm[[y]]$JGeneName==J[x] &
                pt8rpm[[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z])]),
561         unique(pt9rpm[[y]]$cdr3Length[which(pt9rpm[[y]]$JGeneName==J[x] &
                pt9rpm[[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z])])))
```

```
562 #print(L)
563 #if VDJ combination not present set to zero
564 if (L==0){
565 Peaks<-0
566 OligoscoreiGVT<-0
567 OligoscoreiGVHD<-0
568 cbind(Peaks,OligoscoreiGVT,OligoscoreiGVHD)
569 }
570 #this calculates the number of peaks for each subject
571 else {
572 nk=c(length(unique(pt11rpm[[y]]$cdr3Length[which(pt11rpm[[y]]$JGeneName==J[x
      ] & pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName==d[z])])),
573     length(unique(pt12rpm[[y]]$cdr3Length[which(pt12rpm[[y]]$JGeneName==J[x
         ] & pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z])])),
574     length(unique(pt13rpm[[y]]$cdr3Length[which(pt13rpm[[y]]$JGeneName==J[x
         ] & pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z])])),
575     length(unique(pt14rpm[[y]]$cdr3Length[which(pt14rpm[[y]]$JGeneName==J[x
         ] & pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z])])),
576     length(unique(pt16rpm[[y]]$cdr3Length[which(pt16rpm[[y]]$JGeneName==J[x
         ]& pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z])])),
577     length(unique(pt24rpm[[y]]$cdr3Length[which(pt24rpm[[y]]$JGeneName==J[x
         ]& pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z])])),
578     length(unique(pt3rpm[[y]]$cdr3Length[which(pt3rpm[[y]]$JGeneName==J[x]
         & pt3rpm[[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z])])),
579     length(unique(pt4rpm[[y]]$cdr3Length[which(pt4rpm[[y]]$JGeneName==J[x]
         & pt4rpm[[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z])])),
```

```
580     length(unique(pt5rpm[[y]]$cdr3Length[which(pt5rpm[[y]]$JGeneName==J[x]
           & pt5rpm[[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z])])),
581     length(unique(pt6rpm[[y]]$cdr3Length[which(pt6rpm[[y]]$JGeneName==J[x]
           & pt6rpm[[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z])])),
582     length(unique(pt7rpm[[y]]$cdr3Length[which(pt7rpm[[y]]$JGeneName==J[x]
           & pt7rpm[[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z])])),
583     length(unique(pt8rpm[[y]]$cdr3Length[which(pt8rpm[[y]]$JGeneName==J[x]
           & pt8rpm[[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z])])),
584     length(unique(pt9rpm[[y]]$cdr3Length[which(pt9rpm[[y]]$JGeneName==J[x]
           & pt9rpm[[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z])])))
585 #print(nk)
586
587  #This list the peak lengths
588 Peaks=unique(c(unique(pt11rpm[[y]]$cdr3Length[which(pt11rpm[[y]]$JGeneName
       ==J[x] & pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName==d[z])])
       ,
589         unique(pt12rpm[[y]]$cdr3Length[which(pt12rpm[[y]]$JGeneName==J[x] &
                pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z])]),
590         unique(pt13rpm[[y]]$cdr3Length[which(pt13rpm[[y]]$JGeneName==J[x] &
                pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z])]),
591         unique(pt14rpm[[y]]$cdr3Length[which(pt14rpm[[y]]$JGeneName==J[x] &
                pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z])]),
592         unique(pt16rpm[[y]]$cdr3Length[which(pt16rpm[[y]]$JGeneName==J[x]&
               pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z])]),
593         unique(pt24rpm[[y]]$cdr3Length[which(pt24rpm[[y]]$JGeneName==J[x]&
               pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z])]),
```

```
594         unique(pt3rpm[[y]]$cdr3Length[which(pt3rpm[[y]]$JGeneName==J[x] &
               pt3rpm[[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z])]),
595         unique(pt4rpm[[y]]$cdr3Length[which(pt4rpm[[y]]$JGeneName==J[x] &
               pt4rpm[[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z])]),
596         unique(pt5rpm[[y]]$cdr3Length[which(pt5rpm[[y]]$JGeneName==J[x] &
               pt5rpm[[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z])]),
597         unique(pt6rpm[[y]]$cdr3Length[which(pt6rpm[[y]]$JGeneName==J[x] &
               pt6rpm[[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z])]),
598         unique(pt7rpm[[y]]$cdr3Length[which(pt7rpm[[y]]$JGeneName==J[x] &
               pt7rpm[[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z])]),
599         unique(pt8rpm[[y]]$cdr3Length[which(pt8rpm[[y]]$JGeneName==J[x] &
               pt8rpm[[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z])]),
600         unique(pt9rpm[[y]]$cdr3Length[which(pt9rpm[[y]]$JGeneName==J[x] &
               pt9rpm[[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z])])))
601 #print(Peaks)
602 #this calculates the total rpm in each peak
603 Peaksi<-matrix(ncol=13,nrow=L)
604 for (i in 1:L){
605 Peaksi[i,]<-c(sum(pt11rpm[[y]]$RPM[which(pt11rpm[[y]]$JGeneName==J[x] &
      pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName==d[z] & pt11rpm[[y
      ]]$cdr3Length==Peaks[i])]),
606             sum(pt12rpm[[y]]$RPM[which(pt12rpm[[y]]$JGeneName==J[x] &
                   pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z]
                   & pt12rpm[[y]]$cdr3Length==Peaks[i])]),
607             sum(pt13rpm[[y]]$RPM[which(pt13rpm[[y]]$JGeneName==J[x] &
                   pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z]
                   & pt13rpm[[y]]$cdr3Length==Peaks[i])]),
```

```
608             sum(pt14rpm[[y]]$RPM[which(pt14rpm[[y]]$JGeneName==J[x] &
                    pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z]
                    & pt14rpm[[y]]$cdr3Length==Peaks[i])]),
609             sum(pt16rpm[[y]]$RPM[which(pt16rpm[[y]]$JGeneName==J[x] &
                    pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z]
                    & pt16rpm[[y]]$cdr3Length==Peaks[i])]),
610             sum(pt24rpm[[y]]$RPM[which(pt24rpm[[y]]$JGeneName==J[x] &
                    pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z]
                    & pt24rpm[[y]]$cdr3Length==Peaks[i])]),
611             sum(pt3rpm[[y]]$RPM[which(pt3rpm[[y]]$JGeneName==J[x] & pt3rpm
                    [[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z] & pt3rpm
                    [[y]]$cdr3Length==Peaks[i])]),
612             sum(pt4rpm[[y]]$RPM[which(pt4rpm[[y]]$JGeneName==J[x] & pt4rpm
                    [[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z] & pt4rpm
                    [[y]]$cdr3Length==Peaks[i])]),
613             sum(pt5rpm[[y]]$RPM[which(pt5rpm[[y]]$JGeneName==J[x] & pt5rpm
                    [[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z] & pt5rpm
                    [[y]]$cdr3Length==Peaks[i])]),
614             sum(pt6rpm[[y]]$RPM[which(pt6rpm[[y]]$JGeneName==J[x] & pt6rpm
                    [[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z] & pt6rpm
                    [[y]]$cdr3Length==Peaks[i])]),
615             sum(pt7rpm[[y]]$RPM[which(pt7rpm[[y]]$JGeneName==J[x] & pt7rpm
                    [[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z] & pt7rpm
                    [[y]]$cdr3Length==Peaks[i])]),
616             sum(pt8rpm[[y]]$RPM[which(pt8rpm[[y]]$JGeneName==J[x] & pt8rpm
                    [[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z] & pt8rpm
                    [[y]]$cdr3Length==Peaks[i])]),
```

```
617             sum(pt9rpm[[y]]$RPM[which(pt9rpm[[y]]$JGeneName==J[x] & pt9rpm
                    [[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z] & pt9rpm
                    [[y]]$cdr3Length==Peaks[i])]))
618             }
619 #print(Peaksi)
620 #total rpm per patient
621 Peakssum<-apply(Peaksi,2,sum)
622
623 #print(Peakssum)
624 #calculates the probability of each peak
625 Probabilityi<-matrix(ncol=13,nrow=L)
626 for (j in 1:L) {
627 Probabilityi[j,]<-Peaksi[j,]/Peakssum
628 }
629 #sets 0 values and NaN values to a low detection threshold as recommended
630 for (m in 1:L){
631 Probabilityi[m,which(Probabilityi[m,]=="NaN")]<-1e-6
632 Probabilityi[m,which(Probabilityi[m,]==0)]<-1e-6
633 }
634
635 #print(Probabilityi)
636 #calculate oligoscore for GVT group
637 OligoscoreiGVT<-matrix(nrow=L,ncol=1)
638 for (k in 1:L){
639 OligoscoreiGVT[k]<-exp(1)*((prod(Probabilityi[k,GroupGVT]*exp(-nk[GroupGVT])
      ))^(1/length(GroupGVT)))
640 }
```

```
#print(OligoscoreiGVT)
#calculate Oligoscore for GVHD group
OligoscoreiGVHD<-matrix(nrow=L,ncol=1)
for (l in 1:L){
OligoscoreiGVHD[l]<-exp(1)*((prod(Probabilityi[l,GroupGVHD]*exp(-nk[
    GroupGVHD])))^(1/length(GroupGVHD)))
}
#print(OligoscoreiGVHD)

cbind(Peaks,OligoscoreiGVT,OligoscoreiGVHD)
}
}

#make list of VDJ combos
location<-expand.grid(J,V,d)
#calculate oligoscore for each vdj combination
Oligoscores<-matrix(0,ncol=6)
colnames(Oligoscores)<-c("JGeneName","VGeneName","DGeneName","CDR3Length","
    GVT","GVHD")
#a is vdj combination
for (a in 1:1248){
Oligoscoresa<-cbind(location[a,],oligotest(which(J==location[a,1]),which(V==
    location[a,2]),which(d==location[a,3]),2))
colnames(Oligoscoresa)<-c("JGeneName","VGeneName","DGeneName","CDR3Length","
    GVT","GVHD")
Oligoscores<-rbind(Oligoscores,Oligoscoresa)
print(a)
```

```
}

Oligoscores<-Oligoscores[-1,]


setwd("C:/Users/makowski/Desktop/Archer Code and Files")
save(Oligoscores,location,file="Oligoscores.RData")

#run Fisher's exact test
GVHDmatrix<-matrix(c(8,0,2,3),nrow=2,ncol=2)
fisher.test(GVHDmatrix) #p-value = 0.03497
NoGVHDmatrix<-matrix(c(3,5,4,1),nrow=2,ncol=2)
fisher.test(NoGVHDmatrix) #p-value = 0.2657


############################################################
#This section calculated Simpson's Diversity Index
############################################################


setwd("C:/Users/makowski/Desktop/Archer Code and Files")
load("TcellRPM.RData")

#Function that does the Diversity test for the VDJ families x is the J gene,
      w is V gene and z is d gene and y=1 is donor and y=2 is recipient
diversitytest<-function(x,w,z,y){
#this part calculates the number of unique CDR3 lengths
```

```
690 L=length(unique(c(unique(pt11rpm[[y]]$cdr3Length[which(pt11rpm[[y]]$
      JGeneName==J[x] & pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName
      ==d[z])]),
691         unique(pt12rpm[[y]]$cdr3Length[which(pt12rpm[[y]]$JGeneName==J[x] &
                pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z])]),
692         unique(pt13rpm[[y]]$cdr3Length[which(pt13rpm[[y]]$JGeneName==J[x] &
                pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z])]),
693         unique(pt14rpm[[y]]$cdr3Length[which(pt14rpm[[y]]$JGeneName==J[x] &
                pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z])]),
694         unique(pt16rpm[[y]]$cdr3Length[which(pt16rpm[[y]]$JGeneName==J[x]&
               pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z])]),
695         unique(pt24rpm[[y]]$cdr3Length[which(pt24rpm[[y]]$JGeneName==J[x]&
               pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z])]),
696         unique(pt3rpm[[y]]$cdr3Length[which(pt3rpm[[y]]$JGeneName==J[x] &
               pt3rpm[[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z])]),
697         unique(pt4rpm[[y]]$cdr3Length[which(pt4rpm[[y]]$JGeneName==J[x] &
               pt4rpm[[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z])]),
698         unique(pt5rpm[[y]]$cdr3Length[which(pt5rpm[[y]]$JGeneName==J[x] &
               pt5rpm[[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z])]),
699         unique(pt6rpm[[y]]$cdr3Length[which(pt6rpm[[y]]$JGeneName==J[x] &
               pt6rpm[[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z])]),
700         unique(pt7rpm[[y]]$cdr3Length[which(pt7rpm[[y]]$JGeneName==J[x] &
               pt7rpm[[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z])]),
701         unique(pt8rpm[[y]]$cdr3Length[which(pt8rpm[[y]]$JGeneName==J[x] &
               pt8rpm[[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z])]),
702         unique(pt9rpm[[y]]$cdr3Length[which(pt9rpm[[y]]$JGeneName==J[x] &
               pt9rpm[[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z])])))
```

```
703 #print(L)
704 #this sets the perturbation statistic to zero if the VDJ combination is not
        present
705 if (L==0){
706 Diversity<-rep(0,13)
707 Diversity
708 }
709
710 else {
711 #This list the peak lengths
712 Peaks=unique(c(unique(pt11rpm[[y]]$cdr3Length[which(pt11rpm[[y]]$JGeneName
       ==J[x] & pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName==d[z])])
       ,
713         unique(pt12rpm[[y]]$cdr3Length[which(pt12rpm[[y]]$JGeneName==J[x] &
                pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z])]),
714         unique(pt13rpm[[y]]$cdr3Length[which(pt13rpm[[y]]$JGeneName==J[x] &
                pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z])]),
715         unique(pt14rpm[[y]]$cdr3Length[which(pt14rpm[[y]]$JGeneName==J[x] &
                pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z])]),
716         unique(pt16rpm[[y]]$cdr3Length[which(pt16rpm[[y]]$JGeneName==J[x]&
               pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z])]),
717         unique(pt24rpm[[y]]$cdr3Length[which(pt24rpm[[y]]$JGeneName==J[x]&
               pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z])]),
718         unique(pt3rpm[[y]]$cdr3Length[which(pt3rpm[[y]]$JGeneName==J[x] &
               pt3rpm[[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z])]),
719         unique(pt4rpm[[y]]$cdr3Length[which(pt4rpm[[y]]$JGeneName==J[x] &
               pt4rpm[[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z])]),
```

```
720         unique(pt5rpm[[y]]$cdr3Length[which(pt5rpm[[y]]$JGeneName==J[x] &
               pt5rpm[[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z])]),
721         unique(pt6rpm[[y]]$cdr3Length[which(pt6rpm[[y]]$JGeneName==J[x] &
               pt6rpm[[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z])]),
722         unique(pt7rpm[[y]]$cdr3Length[which(pt7rpm[[y]]$JGeneName==J[x] &
               pt7rpm[[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z])]),
723         unique(pt8rpm[[y]]$cdr3Length[which(pt8rpm[[y]]$JGeneName==J[x] &
               pt8rpm[[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z])]),
724         unique(pt9rpm[[y]]$cdr3Length[which(pt9rpm[[y]]$JGeneName==J[x] &
               pt9rpm[[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z])])))
725 #print(Peaks)
726 #this calculates the total rpm in each peak
727 Peaksi<-matrix(ncol=13,nrow=L)
728 for (i in 1:L){
729 Peaksi[i,]<-c(sum(pt11rpm[[y]]$RPM[which(pt11rpm[[y]]$JGeneName==J[x] &
      pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName==d[z] & pt11rpm[[y
      ]]$cdr3Length==Peaks[i])]),
730             sum(pt12rpm[[y]]$RPM[which(pt12rpm[[y]]$JGeneName==J[x] &
                   pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z]
                   & pt12rpm[[y]]$cdr3Length==Peaks[i])]),
731             sum(pt13rpm[[y]]$RPM[which(pt13rpm[[y]]$JGeneName==J[x] &
                   pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z]
                   & pt13rpm[[y]]$cdr3Length==Peaks[i])]),
732             sum(pt14rpm[[y]]$RPM[which(pt14rpm[[y]]$JGeneName==J[x] &
                   pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z]
                   & pt14rpm[[y]]$cdr3Length==Peaks[i])]),
```

```
733             sum(pt16rpm[[y]]$RPM[which(pt16rpm[[y]]$JGeneName==J[x] &
                   pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z]
                   & pt16rpm[[y]]$cdr3Length==Peaks[i])]),
734             sum(pt24rpm[[y]]$RPM[which(pt24rpm[[y]]$JGeneName==J[x] &
                   pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z]
                   & pt24rpm[[y]]$cdr3Length==Peaks[i])]),
735             sum(pt3rpm[[y]]$RPM[which(pt3rpm[[y]]$JGeneName==J[x] & pt3rpm
                   [[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z] & pt3rpm
                   [[y]]$cdr3Length==Peaks[i])]),
736             sum(pt4rpm[[y]]$RPM[which(pt4rpm[[y]]$JGeneName==J[x] & pt4rpm
                   [[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z] & pt4rpm
                   [[y]]$cdr3Length==Peaks[i])]),
737             sum(pt5rpm[[y]]$RPM[which(pt5rpm[[y]]$JGeneName==J[x] & pt5rpm
                   [[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z] & pt5rpm
                   [[y]]$cdr3Length==Peaks[i])]),
738             sum(pt6rpm[[y]]$RPM[which(pt6rpm[[y]]$JGeneName==J[x] & pt6rpm
                   [[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z] & pt6rpm
                   [[y]]$cdr3Length==Peaks[i])]),
739             sum(pt7rpm[[y]]$RPM[which(pt7rpm[[y]]$JGeneName==J[x] & pt7rpm
                   [[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z] & pt7rpm
                   [[y]]$cdr3Length==Peaks[i])]),
740             sum(pt8rpm[[y]]$RPM[which(pt8rpm[[y]]$JGeneName==J[x] & pt8rpm
                   [[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z] & pt8rpm
                   [[y]]$cdr3Length==Peaks[i])]),
741             sum(pt9rpm[[y]]$RPM[which(pt9rpm[[y]]$JGeneName==J[x] & pt9rpm
                   [[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z] & pt9rpm
                   [[y]]$cdr3Length==Peaks[i])]))
```

```
742             }
743 #print(Peaksi)
744  #total rpm per patient
745 Peakssum<-apply(Peaksi,2,sum)
746 #print(Peakssum)
747 #calculates the diversity statistic
748 Diversity<-matrix(nrow=1,ncol=13)
749 for (j in 1:13) {
750 Diversity[j]<-1-sum((Peaksi[,j]*(Peaksi[,j]-1))/(Peakssum[j]*(Peakssum[j]-1)
       ))
751 }
752 #print(Diversity)
753
754 Diversity
755 }
756 }
757 #list of VDJ combinations
758 location<-expand.grid(J,V,d)
759 #calculates diversity statistic for each VDJ combination
760 #a is VDJ combination
761 Diversity<-matrix(nrow=(length(J)*length(V)*length(d)),ncol=13)
762 for (a in 1:1248){
763 Diversity[a,]<-diversitytest(which(J==location[a,1]),which(V==location[a,2])
       ,which(d==location[a,3]),2)
764 print(a)
765 }
766
```

```
767 setwd("C:/Users/makowski/Desktop/Archer Code and Files")
768 save(Diversity,location,file="Diversity.RData")
769
770 ##############################################
771 #Run the permutation tests for the Diversity index
772 ##############################################
773
774
775 GroupGVT<-c(2,5,7,8,11)
776 GroupGVHD<-c(1,3,4,6,9,10,12,13)
777
778 #GVT is 0 and GVHD is 1
779 Group<-c(1,0,1,1,0,1,0,0,1,1,0,1,1)
780
781 #for referencing which VDJ combination
782 rownames(Diversity)<-c(1:1248)
783 #load NA values for tests
784 setwd("C:/Users/makowski/Desktop/Archer Code and Files")
785 load("VDJNAs.RData")
786
787 Diversity.NA<-Diversity[-NAs,]
788 #replace NaN with NA
789 for (i in 1:13){
790   for (j in 1:dim(Diversity.NA)[1]){
791
792 if (Diversity.NA[j,i]=="NaN"){
793 Diversity.NA[j,i]<-NA
```

```
}
}
} }

set.seed(123)
B <- 1000
test.stat<-pval<-numeric()
test.stat.b<-matrix(nrow=dim(Diversity.NA)[1], ncol=B)
for (i in 1:dim(Diversity.NA)[1]) {

        test.stat[i]<-mean(Diversity.NA[i,Group==1],na.rm=TRUE) - mean(
           Diversity.NA[i,Group==0],na.rm=TRUE)
         for (j in 1:B) {

                sample<-sample(Group, replace=FALSE)
                 test.stat.b[i,j]<-mean(Diversity.NA[i,sample==1],na.rm=TRUE)
                      - mean(Diversity.NA[i,sample==0],na.rm=TRUE)
         }
         pval[i]<-sum(abs(test.stat.b[i,]>abs(test.stat[i])))/B
}

fdr<-cbind(p.adjust(pval, method="BH"),c(1:dim(Diversity.NA)[1]))
length(which(fdr[,1]<0.05))
fdr.order<-fdr[order(pval),]
location.NA<-location[-NAs,]
groupnames<-factor(Group,levels=c(0,1),labels=c("No GVHD","GVHD"))
#make some plots
```

```
819 png(file = "Diversitytest.png", width = 1200, height = 1000, units = "px")
820 par(mfrow=c(3,2),mar=c(5.1,4.5,4.1,2.1))
821 plot(groupnames,Diversity.NA[fdr.order[1,2],],main=paste(location.NA[fdr.
      order[1,2],1],location.NA[fdr.order[1,2],2],location.NA[fdr.order
      [1,2],3]),cex.lab=2,cex.main=2,cex.axis=2,ylab="Diversity")
822 plot(groupnames,Diversity.NA[fdr.order[2,2],],main=paste(location.NA[fdr.
      order[2,2],1],location.NA[fdr.order[2,2],2],location.NA[fdr.order
      [2,2],3]),cex.lab=2,cex.main=2,cex.axis=2,ylab="Diversity")
823 plot(groupnames,Diversity.NA[fdr.order[3,2],],main=paste(location.NA[fdr.
      order[3,2],1],location.NA[fdr.order[3,2],2],location.NA[fdr.order
      [3,2],3]),cex.lab=2,cex.main=2,cex.axis=2,ylab="Diversity")
824 plot(groupnames,Diversity.NA[fdr.order[4,2],],main=paste(location.NA[fdr.
      order[4,2],1],location.NA[fdr.order[4,2],2],location.NA[fdr.order
      [4,2],3]),cex.lab=2,cex.main=2,cex.axis=2,ylab="Diversity")
825 plot(groupnames,Diversity.NA[fdr.order[5,2],],main=paste(location.NA[fdr.
      order[5,2],1],location.NA[fdr.order[5,2],2],location.NA[fdr.order
      [5,2],3]),cex.lab=2,cex.main=2,cex.axis=2,ylab="Diversity")
826 dev.off()
827
828
829 library(xtable)
830 apply(t(Diversity.NA[fdr.order[c(1:5),2],GroupGVT]),2,FUN=median,na.rm=TRUE)
831 perttest.table<-cbind(location.NA[fdr.order[c(1:5),2],],apply(t(Diversity.NA
      [fdr.order[c(1:5),2],GroupGVT]),2,FUN=median,na.rm=TRUE),apply(t(
      Diversity.NA[fdr.order[c(1:5),2],GroupGVHD]),2,FUN=median,na.rm=TRUE),
      fdr.order[c(1:5),1])
```

```
colnames(perttest.table)<-c("JGene","VGene","DGene", "Median No GVHD
    Perturbation", "Median GVHD Perturbation", "FDR")
xtable(perttest.table,digits=3)


##########################################################
#This section calculated Shannon Diversity Index
##########################################################

 setwd("C:/Users/makowski/Desktop/Archer Code and Files")
load("TcellRPM.RData")

#Function that does the Diversity test for the VDJ families x is the J gene,
     w is V gene and z is d gene and y=1 is donor and y=2 is recipient
diversitytest<-function(x,w,z,y){
#this part calculates the number of unique CDR3 lengths
L=length(unique(c(unique(pt11rpm[[y]]$cdr3Length[which(pt11rpm[[y]]$
    JGeneName==J[x] & pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName
    ==d[z])]),
        unique(pt12rpm[[y]]$cdr3Length[which(pt12rpm[[y]]$JGeneName==J[x] &
            pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z])]),
        unique(pt13rpm[[y]]$cdr3Length[which(pt13rpm[[y]]$JGeneName==J[x] &
            pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z])]),
        unique(pt14rpm[[y]]$cdr3Length[which(pt14rpm[[y]]$JGeneName==J[x] &
            pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z])]),
```

```
852         unique(pt16rpm[[y]]$cdr3Length[which(pt16rpm[[y]]$JGeneName==J[x]&
               pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z])]),
853         unique(pt24rpm[[y]]$cdr3Length[which(pt24rpm[[y]]$JGeneName==J[x]&
               pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z])]),
854         unique(pt3rpm[[y]]$cdr3Length[which(pt3rpm[[y]]$JGeneName==J[x] &
               pt3rpm[[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z])]),
855         unique(pt4rpm[[y]]$cdr3Length[which(pt4rpm[[y]]$JGeneName==J[x] &
               pt4rpm[[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z])]),
856         unique(pt5rpm[[y]]$cdr3Length[which(pt5rpm[[y]]$JGeneName==J[x] &
               pt5rpm[[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z])]),
857         unique(pt6rpm[[y]]$cdr3Length[which(pt6rpm[[y]]$JGeneName==J[x] &
               pt6rpm[[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z])]),
858         unique(pt7rpm[[y]]$cdr3Length[which(pt7rpm[[y]]$JGeneName==J[x] &
               pt7rpm[[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z])]),
859         unique(pt8rpm[[y]]$cdr3Length[which(pt8rpm[[y]]$JGeneName==J[x] &
               pt8rpm[[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z])]),
860         unique(pt9rpm[[y]]$cdr3Length[which(pt9rpm[[y]]$JGeneName==J[x] &
               pt9rpm[[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z])])))
861 #print(L)
862 #this sets the perturbation statistic to zero if the VDJ combination is not
        present
863 if (L==0){
864 Diversity<-rep(0,13)
865 Diversity
866 }
867
868 else {
```

```
869 #This list the peak lengths
870 Peaks=unique(c(unique(pt11rpm[[y]]$cdr3Length[which(pt11rpm[[y]]$JGeneName
       ==J[x] & pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName==d[z])])
       ,
871         unique(pt12rpm[[y]]$cdr3Length[which(pt12rpm[[y]]$JGeneName==J[x] &
                pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z])]),
872         unique(pt13rpm[[y]]$cdr3Length[which(pt13rpm[[y]]$JGeneName==J[x] &
                pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z])]),
873         unique(pt14rpm[[y]]$cdr3Length[which(pt14rpm[[y]]$JGeneName==J[x] &
                pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z])]),
874         unique(pt16rpm[[y]]$cdr3Length[which(pt16rpm[[y]]$JGeneName==J[x]&
               pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z])]),
875         unique(pt24rpm[[y]]$cdr3Length[which(pt24rpm[[y]]$JGeneName==J[x]&
               pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z])]),
876         unique(pt3rpm[[y]]$cdr3Length[which(pt3rpm[[y]]$JGeneName==J[x] &
               pt3rpm[[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z])]),
877         unique(pt4rpm[[y]]$cdr3Length[which(pt4rpm[[y]]$JGeneName==J[x] &
               pt4rpm[[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z])]),
878         unique(pt5rpm[[y]]$cdr3Length[which(pt5rpm[[y]]$JGeneName==J[x] &
               pt5rpm[[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z])]),
879         unique(pt6rpm[[y]]$cdr3Length[which(pt6rpm[[y]]$JGeneName==J[x] &
               pt6rpm[[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z])]),
880         unique(pt7rpm[[y]]$cdr3Length[which(pt7rpm[[y]]$JGeneName==J[x] &
               pt7rpm[[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z])]),
881         unique(pt8rpm[[y]]$cdr3Length[which(pt8rpm[[y]]$JGeneName==J[x] &
               pt8rpm[[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z])]),
```

```
882         unique(pt9rpm[[y]]$cdr3Length[which(pt9rpm[[y]]$JGeneName==J[x] &
               pt9rpm[[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z])])))
883 #print(Peaks)
884 #this calculates the total rpm in each peak
885 Peaksi<-matrix(ncol=13,nrow=L)
886 for (i in 1:L){
887 Peaksi[i,]<-c(sum(pt11rpm[[y]]$RPM[which(pt11rpm[[y]]$JGeneName==J[x] &
      pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName==d[z] & pt11rpm[[y
      ]]$cdr3Length==Peaks[i])]),
888             sum(pt12rpm[[y]]$RPM[which(pt12rpm[[y]]$JGeneName==J[x] &
                   pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z]
                   & pt12rpm[[y]]$cdr3Length==Peaks[i])]),
889             sum(pt13rpm[[y]]$RPM[which(pt13rpm[[y]]$JGeneName==J[x] &
                   pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z]
                   & pt13rpm[[y]]$cdr3Length==Peaks[i])]),
890             sum(pt14rpm[[y]]$RPM[which(pt14rpm[[y]]$JGeneName==J[x] &
                   pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z]
                   & pt14rpm[[y]]$cdr3Length==Peaks[i])]),
891             sum(pt16rpm[[y]]$RPM[which(pt16rpm[[y]]$JGeneName==J[x] &
                   pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z]
                   & pt16rpm[[y]]$cdr3Length==Peaks[i])]),
892             sum(pt24rpm[[y]]$RPM[which(pt24rpm[[y]]$JGeneName==J[x] &
                   pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z]
                   & pt24rpm[[y]]$cdr3Length==Peaks[i])]),
893             sum(pt3rpm[[y]]$RPM[which(pt3rpm[[y]]$JGeneName==J[x] & pt3rpm
                   [[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z] & pt3rpm
                   [[y]]$cdr3Length==Peaks[i])]),
```

```
894             sum(pt4rpm[[y]]$RPM[which(pt4rpm[[y]]$JGeneName==J[x] & pt4rpm
                   [[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z] & pt4rpm
                   [[y]]$cdr3Length==Peaks[i])]),
895             sum(pt5rpm[[y]]$RPM[which(pt5rpm[[y]]$JGeneName==J[x] & pt5rpm
                   [[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z] & pt5rpm
                   [[y]]$cdr3Length==Peaks[i])]),
896             sum(pt6rpm[[y]]$RPM[which(pt6rpm[[y]]$JGeneName==J[x] & pt6rpm
                   [[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z] & pt6rpm
                   [[y]]$cdr3Length==Peaks[i])]),
897             sum(pt7rpm[[y]]$RPM[which(pt7rpm[[y]]$JGeneName==J[x] & pt7rpm
                   [[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z] & pt7rpm
                   [[y]]$cdr3Length==Peaks[i])]),
898             sum(pt8rpm[[y]]$RPM[which(pt8rpm[[y]]$JGeneName==J[x] & pt8rpm
                   [[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z] & pt8rpm
                   [[y]]$cdr3Length==Peaks[i])]),
899             sum(pt9rpm[[y]]$RPM[which(pt9rpm[[y]]$JGeneName==J[x] & pt9rpm
                   [[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z] & pt9rpm
                   [[y]]$cdr3Length==Peaks[i])]))
900             }
901 #print(Peaksi)
902  #total rpm per patient
903 Peakssum<-apply(Peaksi,2,sum)
904 #print(Peakssum)
905 #calculates the diversity statistic
906 Diversity<-matrix(nrow=1,ncol=13)
907 for (j in 1:13) {
908 Diversity[j]<-(-1)*sum((Peaksi[,j]*log(Peaksi[,j])),na.rm=TRUE)
```

```
}
#print(Diversity)

Diversity
}
}
#list of VDJ combinations
location<-expand.grid(J,V,d)
#calculates diversity statistic for each VDJ combination
#a is VDJ combination
Diversity<-matrix(nrow=(length(J)*length(V)*length(d)),ncol=13)
for (a in 1:1248){
Diversity[a,]<-diversitytest(which(J==location[a,1]),which(V==location[a,2])
    ,which(d==location[a,3]),2)
print(a)
}

save(Diversity,location,file="ShannaonDiversity.RData")


##############################################
#Run the permutation tests for the Diversity index
##############################################

GroupGVT<-c(2,5,7,8,11)
GroupGVHD<-c(1,3,4,6,9,10,12,13)

```

```
#GVT is 0 and GVHD is 1
Group<-c(1,0,1,1,0,1,0,0,1,1,0,1,1)

#for referencing which VDJ combination
rownames(Diversity)<-c(1:1248)
#load NA values for tests
setwd("C:/Users/makowski/Desktop/Archer Code and Files")
load("VDJNAs.RData")

Diversity.NA<-Diversity[-NAs,] }

set.seed(123)
B <- 1000
test.stat<-pval<-numeric()
test.stat.b<-matrix(nrow=dim(Diversity.NA)[1], ncol=B)
for (i in 1:dim(Diversity.NA)[1]) {

       test.stat[i]<-mean(Diversity.NA[i,Group==1],na.rm=TRUE) - mean(
          Diversity.NA[i,Group==0],na.rm=TRUE)
        for (j in 1:B) {

              sample<-sample(Group, replace=FALSE)
               test.stat.b[i,j]<-mean(Diversity.NA[i,sample==1],na.rm=TRUE)
                   - mean(Diversity.NA[i,sample==0],na.rm=TRUE)
        }
        pval[i]<-sum(abs(test.stat.b[i,]>abs(test.stat[i])))/B
}
```

```
960
961 fdr<-cbind(p.adjust(pval, method="BH"),c(1:dim(Diversity.NA)[1]))
962 length(which(fdr[,1]<0.05))
963 fdr.order<-fdr[order(pval),]
964 location.NA<-location[-NAs,]
965
966 groupnames<-factor(Group,levels=c(0,1),labels=c("No GVHD","GVHD"))
967 #make some plots
968 png(file = "DiversityShannon.png", width = 1200, height = 1000, units = "px"
      )
969 par(mfrow=c(3,2),mar=c(5.1,4.5,4.1,2.1))
970 plot(groupnames,Diversity.NA[fdr.order[1,2],],main=paste(location.NA[fdr.
      order[1,2],1],location.NA[fdr.order[1,2],2],location.NA[fdr.order
      [1,2],3]),cex.lab=2,cex.main=2,cex.axis=2,ylab="Diversity")
971 plot(groupnames,Diversity.NA[fdr.order[2,2],],main=paste(location.NA[fdr.
      order[2,2],1],location.NA[fdr.order[2,2],2],location.NA[fdr.order
      [2,2],3]),cex.lab=2,cex.main=2,cex.axis=2,ylab="Diversity")
972 plot(groupnames,Diversity.NA[fdr.order[3,2],],main=paste(location.NA[fdr.
      order[3,2],1],location.NA[fdr.order[3,2],2],location.NA[fdr.order
      [3,2],3]),cex.lab=2,cex.main=2,cex.axis=2,ylab="Diversity")
973 plot(groupnames,Diversity.NA[fdr.order[4,2],],main=paste(location.NA[fdr.
      order[4,2],1],location.NA[fdr.order[4,2],2],location.NA[fdr.order
      [4,2],3]),cex.lab=2,cex.main=2,cex.axis=2,ylab="Diversity")
974 plot(groupnames,Diversity.NA[fdr.order[5,2],],main=paste(location.NA[fdr.
      order[5,2],1],location.NA[fdr.order[5,2],2],location.NA[fdr.order
      [5,2],3]),cex.lab=2,cex.main=2,cex.axis=2,ylab="Diversity")
```

```
975 plot(groupnames,Diversity.NA[fdr.order[6,2],],main=paste(location.NA[fdr.
       order[6,2],1],location.NA[fdr.order[6,2],2],location.NA[fdr.order
       [6,2],3]),cex.lab=2,cex.main=2,cex.axis=2,ylab="Diversity")
976 dev.off()
977
978
979 library(xtable)
980 apply(t(Diversity.NA[fdr.order[c(1:5),2],GroupGVT]),2,FUN=median,na.rm=TRUE)
981 perttest.table<-cbind(location.NA[fdr.order[c(1:6),2],],apply(t(Diversity.NA
       [fdr.order[c(1:6),2],GroupGVT]),2,FUN=median,na.rm=TRUE),apply(t(
       Diversity.NA[fdr.order[c(1:6),2],GroupGVHD]),2,FUN=median,na.rm=TRUE),
       pval[fdr.order[c(1:6),2]])
982 colnames(perttest.table)<-c("JGene","VGene","DGene", "Median No GVHD
       Perturbation", "Median GVHD Perturbation", "p-value")
983 xtable(perttest.table,digits=3)
984
985
986
987 ############################################################
988 #This section computes tke K-L Divergence test statistics
989 ############################################################
990
991 setwd("C:/Users/makowski/Desktop/Archer Code and Files")
992 load("TcellRPM.RData")
993
994 #Function that does the distribution test for the VDJ families x is the J
       gene v is the Vgene and w is the d gene and y=1 is donor and y=2 is
```

```
    recipient
995 VDJKLtest<-function(x,v,w,y){
996 #this part calculates the number of unique CDR3 lengths
997 L=length(unique(c(unique(pt11rpm[[y]]$cdr3Length[which(pt11rpm[[y]]$
    JGeneName==J[x] & pt11rpm[[y]]$VGeneName==V[v] & pt11rpm[[y]]$DGeneName
    ==d[w])]),
998         unique(pt12rpm[[y]]$cdr3Length[which(pt12rpm[[y]]$JGeneName==J[x] &
              pt12rpm[[y]]$VGeneName==V[v] & pt12rpm[[y]]$DGeneName==d[w])]),
999         unique(pt13rpm[[y]]$cdr3Length[which(pt13rpm[[y]]$JGeneName==J[x] &
              pt13rpm[[y]]$VGeneName==V[v] & pt13rpm[[y]]$DGeneName==d[w])]),
1000        unique(pt14rpm[[y]]$cdr3Length[which(pt14rpm[[y]]$JGeneName==J[x] &
              pt14rpm[[y]]$VGeneName==V[v] & pt14rpm[[y]]$DGeneName==d[w])]),
1001        unique(pt16rpm[[y]]$cdr3Length[which(pt16rpm[[y]]$JGeneName==J[x] &
              pt16rpm[[y]]$VGeneName==V[v] & pt16rpm[[y]]$DGeneName==d[w])]),
1002        unique(pt24rpm[[y]]$cdr3Length[which(pt24rpm[[y]]$JGeneName==J[x] &
              pt24rpm[[y]]$VGeneName==V[v] & pt24rpm[[y]]$DGeneName==d[w])]),
1003        unique(pt3rpm[[y]]$cdr3Length[which(pt3rpm[[y]]$JGeneName==J[x] &
             pt3rpm[[y]]$VGeneName==V[v] & pt3rpm[[y]]$DGeneName==d[w])]),
1004        unique(pt4rpm[[y]]$cdr3Length[which(pt4rpm[[y]]$JGeneName==J[x] &
             pt4rpm[[y]]$VGeneName==V[v] & pt4rpm[[y]]$DGeneName==d[w])]),
1005        unique(pt5rpm[[y]]$cdr3Length[which(pt5rpm[[y]]$JGeneName==J[x] &
             pt5rpm[[y]]$VGeneName==V[v] & pt5rpm[[y]]$DGeneName==d[w])]),
1006        unique(pt6rpm[[y]]$cdr3Length[which(pt6rpm[[y]]$JGeneName==J[x] &
             pt6rpm[[y]]$VGeneName==V[v] & pt6rpm[[y]]$DGeneName==d[w])]),
1007        unique(pt7rpm[[y]]$cdr3Length[which(pt7rpm[[y]]$JGeneName==J[x] &
             pt7rpm[[y]]$VGeneName==V[v] & pt7rpm[[y]]$DGeneName==d[w])]),
```

```
1008        unique(pt8rpm[[y]]$cdr3Length[which(pt8rpm[[y]]$JGeneName==J[x] &
               pt8rpm[[y]]$VGeneName==V[v] & pt8rpm[[y]]$DGeneName==d[w])]),
1009        unique(pt9rpm[[y]]$cdr3Length[which(pt9rpm[[y]]$JGeneName==J[x] &
               pt9rpm[[y]]$VGeneName==V[v] & pt9rpm[[y]]$DGeneName==d[w])])))
1010        #print(L)
1011
1012 #files2 <- ls(pattern="rpm$")
1013 #files2<-files2[c(-1,-15)]
1014 #keeps only VDJ combinations that have at least 2 peaks
1015  if (L<2){
1016 VDJdist<-c("NA","NA","NA")
1017 list(VDJdist[1],VDJdist[2],VDJdist[3])
1018 }
1019
1020 else {
1021
1022 #This lists the CDR3 lengths
1023 cdr3L<-unique(c(unique(pt11rpm[[y]]$cdr3Length[which(pt11rpm[[y]]$JGeneName
       ==J[x] & pt11rpm[[y]]$VGeneName==V[v] & pt11rpm[[y]]$DGeneName==d[w])])
       ,
1024        unique(pt12rpm[[y]]$cdr3Length[which(pt12rpm[[y]]$JGeneName==J[x] &
               pt12rpm[[y]]$VGeneName==V[v] & pt12rpm[[y]]$DGeneName==d[w])]),
1025        unique(pt13rpm[[y]]$cdr3Length[which(pt13rpm[[y]]$JGeneName==J[x] &
               pt13rpm[[y]]$VGeneName==V[v] & pt13rpm[[y]]$DGeneName==d[w])]),
1026        unique(pt14rpm[[y]]$cdr3Length[which(pt14rpm[[y]]$JGeneName==J[x] &
               pt14rpm[[y]]$VGeneName==V[v] & pt14rpm[[y]]$DGeneName==d[w])]),
```

```
1027          unique(pt16rpm[[y]]$cdr3Length[which(pt16rpm[[y]]$JGeneName==J[x] &
                  pt16rpm[[y]]$VGeneName==V[v] & pt16rpm[[y]]$DGeneName==d[w])]),
1028          unique(pt24rpm[[y]]$cdr3Length[which(pt24rpm[[y]]$JGeneName==J[x] &
                  pt24rpm[[y]]$VGeneName==V[v] & pt24rpm[[y]]$DGeneName==d[w])]),
1029          unique(pt3rpm[[y]]$cdr3Length[which(pt3rpm[[y]]$JGeneName==J[x] &
                  pt3rpm[[y]]$VGeneName==V[v] & pt3rpm[[y]]$DGeneName==d[w])]),
1030          unique(pt4rpm[[y]]$cdr3Length[which(pt4rpm[[y]]$JGeneName==J[x] &
                  pt4rpm[[y]]$VGeneName==V[v] & pt4rpm[[y]]$DGeneName==d[w])]),
1031          unique(pt5rpm[[y]]$cdr3Length[which(pt5rpm[[y]]$JGeneName==J[x] &
                  pt5rpm[[y]]$VGeneName==V[v] & pt5rpm[[y]]$DGeneName==d[w])]),
1032          unique(pt6rpm[[y]]$cdr3Length[which(pt6rpm[[y]]$JGeneName==J[x] &
                  pt6rpm[[y]]$VGeneName==V[v] & pt6rpm[[y]]$DGeneName==d[w])]),
1033          unique(pt7rpm[[y]]$cdr3Length[which(pt7rpm[[y]]$JGeneName==J[x] &
                  pt7rpm[[y]]$VGeneName==V[v] & pt7rpm[[y]]$DGeneName==d[w])]),
1034          unique(pt8rpm[[y]]$cdr3Length[which(pt8rpm[[y]]$JGeneName==J[x] &
                  pt8rpm[[y]]$VGeneName==V[v] & pt8rpm[[y]]$DGeneName==d[w])]),
1035          unique(pt9rpm[[y]]$cdr3Length[which(pt9rpm[[y]]$JGeneName==J[x] &
                  pt9rpm[[y]]$VGeneName==V[v] & pt9rpm[[y]]$DGeneName==d[w])])))
1036
1037 #this calculates an overall abundance for each CDR3 length
1038 overall<-function(z){
1039 sum(sum(pt11rpm[[y]]$RPM[which(pt11rpm[[y]]$JGeneName==J[x] & pt11rpm[[y]]$
       cdr3Length==cdr3L[z] & pt11rpm[[y]]$VGeneName==V[v] & pt11rpm[[y]]$
       DGeneName==d[w])]),
1040     sum(pt12rpm[[y]]$RPM[which(pt12rpm[[y]]$JGeneName==J[x] & pt12rpm[[y]]$
           cdr3Length==cdr3L[z] & pt12rpm[[y]]$VGeneName==V[v] & pt12rpm[[y]]$
           DGeneName==d[w])]),
```

```
1041    sum(pt13rpm[[y]]$RPM[which(pt13rpm[[y]]$JGeneName==J[x] & pt13rpm[[y]]$
           cdr3Length==cdr3L[z] & pt13rpm[[y]]$VGeneName==V[v] & pt13rpm[[y]]$
           DGeneName==d[w])]),
1042    sum(pt14rpm[[y]]$RPM[which(pt14rpm[[y]]$JGeneName==J[x] & pt14rpm[[y]]$
           cdr3Length==cdr3L[z] & pt14rpm[[y]]$VGeneName==V[v] & pt14rpm[[y]]$
           DGeneName==d[w])]),
1043    sum(pt16rpm[[y]]$RPM[which(pt16rpm[[y]]$JGeneName==J[x] & pt16rpm[[y]]$
           cdr3Length==cdr3L[z] & pt16rpm[[y]]$VGeneName==V[v] & pt16rpm[[y]]$
           DGeneName==d[w])]),
1044    sum(pt24rpm[[y]]$RPM[which(pt24rpm[[y]]$JGeneName==J[x] & pt24rpm[[y]]$
           cdr3Length==cdr3L[z] & pt24rpm[[y]]$VGeneName==V[v] & pt24rpm[[y]]$
           DGeneName==d[w])]),
1045    sum(pt3rpm[[y]]$RPM[which(pt3rpm[[y]]$JGeneName==J[x] & pt3rpm[[y]]$
           cdr3Length==cdr3L[z] & pt3rpm[[y]]$VGeneName==V[v] & pt3rpm[[y]]$
           DGeneName==d[w])]),
1046    sum(pt4rpm[[y]]$RPM[which(pt4rpm[[y]]$JGeneName==J[x] & pt4rpm[[y]]$
           cdr3Length==cdr3L[z] & pt4rpm[[y]]$VGeneName==V[v] & pt4rpm[[y]]$
           DGeneName==d[w])]),
1047    sum(pt5rpm[[y]]$RPM[which(pt5rpm[[y]]$JGeneName==J[x] & pt5rpm[[y]]$
           cdr3Length==cdr3L[z] & pt5rpm[[y]]$VGeneName==V[v] & pt5rpm[[y]]$
           DGeneName==d[w])]),
1048    sum(pt6rpm[[y]]$RPM[which(pt6rpm[[y]]$JGeneName==J[x] & pt6rpm[[y]]$
           cdr3Length==cdr3L[z] & pt6rpm[[y]]$VGeneName==V[v] & pt6rpm[[y]]$
           DGeneName==d[w])]),
1049    sum(pt7rpm[[y]]$RPM[which(pt7rpm[[y]]$JGeneName==J[x] & pt7rpm[[y]]$
           cdr3Length==cdr3L[z] & pt7rpm[[y]]$VGeneName==V[v] & pt7rpm[[y]]$
           DGeneName==d[w])]),
```

```
1050     sum(pt8rpm[[y]]$RPM[which(pt8rpm[[y]]$JGeneName==J[x] & pt8rpm[[y]]$
             cdr3Length==cdr3L[z] & pt8rpm[[y]]$VGeneName==V[v] & pt8rpm[[y]]$
             DGeneName==d[w])]),
1051     sum(pt9rpm[[y]]$RPM[which(pt9rpm[[y]]$JGeneName==J[x] & pt9rpm[[y]]$
             cdr3Length==cdr3L[z] & pt9rpm[[y]]$VGeneName==V[v] & pt9rpm[[y]]$
             DGeneName==d[w])]))
1052     }
1053
1054 #create matrix to store all these values
1055 rbar<-matrix(nrow=L)
1056
1057 for (i in 1:L){
1058   rbar[i]<-overall(i)/13
1059   }
1060 #print(rbar)
1061
1062 #these two functions calculate the relative abundances for each group
1063 GVT<-function(z){
1064 sum(sum(pt12rpm[[y]]$RPM[which(pt12rpm[[y]]$JGeneName==J[x] & pt12rpm[[y]]$
        cdr3Length==cdr3L[z] & pt12rpm[[y]]$VGeneName==V[v] & pt12rpm[[y]]$
        DGeneName==d[w])]),
1065     sum(pt16rpm[[y]]$RPM[which(pt16rpm[[y]]$JGeneName==J[x] & pt16rpm[[y]]$
             cdr3Length==cdr3L[z] & pt16rpm[[y]]$VGeneName==V[v] & pt16rpm[[y]]$
             DGeneName==d[w])]),
1066     sum(pt3rpm[[y]]$RPM[which(pt3rpm[[y]]$JGeneName==J[x] & pt3rpm[[y]]$
             cdr3Length==cdr3L[z] & pt3rpm[[y]]$VGeneName==V[v] & pt3rpm[[y]]$
             DGeneName==d[w])]),
```

```
1067     sum(pt4rpm[[y]]$RPM[which(pt4rpm[[y]]$JGeneName==J[x] & pt4rpm[[y]]$
             cdr3Length==cdr3L[z] & pt4rpm[[y]]$VGeneName==V[v] & pt4rpm[[y]]$
             DGeneName==d[w])]),
1068     sum(pt7rpm[[y]]$RPM[which(pt7rpm[[y]]$JGeneName==J[x] & pt7rpm[[y]]$
             cdr3Length==cdr3L[z] & pt7rpm[[y]]$VGeneName==V[v] & pt7rpm[[y]]$
             DGeneName==d[w])]))
1069     }
1070
1071 GVHD<-function(z){
1072 sum(sum(pt11rpm[[y]]$RPM[which(pt11rpm[[y]]$JGeneName==J[x] & pt11rpm[[y]]$
        cdr3Length==cdr3L[z] & pt11rpm[[y]]$VGeneName==V[v] & pt11rpm[[y]]$
        DGeneName==d[w])]),
1073     sum(pt13rpm[[y]]$RPM[which(pt13rpm[[y]]$JGeneName==J[x] & pt13rpm[[y]]$
             cdr3Length==cdr3L[z] & pt13rpm[[y]]$VGeneName==V[v] & pt13rpm[[y]]$
             DGeneName==d[w])]),
1074     sum(pt14rpm[[y]]$RPM[which(pt14rpm[[y]]$JGeneName==J[x] & pt14rpm[[y]]$
             cdr3Length==cdr3L[z] & pt14rpm[[y]]$VGeneName==V[v] & pt14rpm[[y]]$
             DGeneName==d[w])]),
1075     sum(pt24rpm[[y]]$RPM[which(pt24rpm[[y]]$JGeneName==J[x] & pt24rpm[[y]]$
             cdr3Length==cdr3L[z] & pt24rpm[[y]]$VGeneName==V[v] & pt24rpm[[y]]$
             DGeneName==d[w])]),
1076     sum(pt5rpm[[y]]$RPM[which(pt5rpm[[y]]$JGeneName==J[x] & pt5rpm[[y]]$
             cdr3Length==cdr3L[z] & pt5rpm[[y]]$VGeneName==V[v] & pt5rpm[[y]]$
             DGeneName==d[w])]),
1077     sum(pt6rpm[[y]]$RPM[which(pt6rpm[[y]]$JGeneName==J[x] & pt6rpm[[y]]$
             cdr3Length==cdr3L[z] & pt6rpm[[y]]$VGeneName==V[v] & pt6rpm[[y]]$
             DGeneName==d[w])]),
```

```
1078     sum(pt8rpm[[y]]$RPM[which(pt8rpm[[y]]$JGeneName==J[x] & pt8rpm[[y]]$
             cdr3Length==cdr3L[z] & pt8rpm[[y]]$VGeneName==V[v] & pt8rpm[[y]]$
             DGeneName==d[w])]),
1079     sum(pt9rpm[[y]]$RPM[which(pt9rpm[[y]]$JGeneName==J[x] & pt9rpm[[y]]$
             cdr3Length==cdr3L[z] & pt9rpm[[y]]$VGeneName==V[v] & pt9rpm[[y]]$
             DGeneName==d[w])]))
1080     }
1081
1082 G=2
1083 #get average for each group
1084 rgroup<-matrix(nrow=L,ncol=G)
1085 for (i in 1:L){
1086   rgroup[i,1]<-GVT(i)/5
1087   rgroup[i,2]<-GVHD(i)/8
1088   }
1089 #print(rgroup)
1090 #this calculated abundances for each subject
1091 indiv<-function(z){
1092 cbind(sum(pt11rpm[[y]]$RPM[which(pt11rpm[[y]]$JGeneName==J[x] & pt11rpm[[y]]
        $cdr3Length==cdr3L[z] & pt11rpm[[y]]$VGeneName==V[v] & pt11rpm[[y]]$
        DGeneName==d[w])]),
1093     sum(pt12rpm[[y]]$RPM[which(pt12rpm[[y]]$JGeneName==J[x] & pt12rpm[[y]]$
             cdr3Length==cdr3L[z] & pt12rpm[[y]]$VGeneName==V[v] & pt12rpm[[y]]$
             DGeneName==d[w])]),
1094     sum(pt13rpm[[y]]$RPM[which(pt13rpm[[y]]$JGeneName==J[x] & pt13rpm[[y]]$
             cdr3Length==cdr3L[z] & pt13rpm[[y]]$VGeneName==V[v] & pt13rpm[[y]]$
             DGeneName==d[w])]),
```

```
1095    sum(pt14rpm[[y]]$RPM[which(pt14rpm[[y]]$JGeneName==J[x] & pt14rpm[[y]]$
           cdr3Length==cdr3L[z] & pt14rpm[[y]]$VGeneName==V[v] & pt14rpm[[y]]$
           DGeneName==d[w])]),
1096    sum(pt16rpm[[y]]$RPM[which(pt16rpm[[y]]$JGeneName==J[x] & pt16rpm[[y]]$
           cdr3Length==cdr3L[z] & pt16rpm[[y]]$VGeneName==V[v] & pt16rpm[[y]]$
           DGeneName==d[w])]),
1097    sum(pt24rpm[[y]]$RPM[which(pt24rpm[[y]]$JGeneName==J[x] & pt24rpm[[y]]$
           cdr3Length==cdr3L[z] & pt24rpm[[y]]$VGeneName==V[v] & pt24rpm[[y]]$
           DGeneName==d[w])]),
1098    sum(pt3rpm[[y]]$RPM[which(pt3rpm[[y]]$JGeneName==J[x] & pt3rpm[[y]]$
           cdr3Length==cdr3L[z] & pt3rpm[[y]]$VGeneName==V[v] & pt3rpm[[y]]$
           DGeneName==d[w])]),
1099    sum(pt4rpm[[y]]$RPM[which(pt4rpm[[y]]$JGeneName==J[x] & pt4rpm[[y]]$
           cdr3Length==cdr3L[z] & pt4rpm[[y]]$VGeneName==V[v] & pt4rpm[[y]]$
           DGeneName==d[w])]),
1100    sum(pt5rpm[[y]]$RPM[which(pt5rpm[[y]]$JGeneName==J[x] & pt5rpm[[y]]$
           cdr3Length==cdr3L[z] & pt5rpm[[y]]$VGeneName==V[v] & pt5rpm[[y]]$
           DGeneName==d[w])]),
1101    sum(pt6rpm[[y]]$RPM[which(pt6rpm[[y]]$JGeneName==J[x] & pt6rpm[[y]]$
           cdr3Length==cdr3L[z] & pt6rpm[[y]]$VGeneName==V[v] & pt6rpm[[y]]$
           DGeneName==d[w])]),
1102    sum(pt7rpm[[y]]$RPM[which(pt7rpm[[y]]$JGeneName==J[x] & pt7rpm[[y]]$
           cdr3Length==cdr3L[z] & pt7rpm[[y]]$VGeneName==V[v] & pt7rpm[[y]]$
           DGeneName==d[w])]),
1103    sum(pt8rpm[[y]]$RPM[which(pt8rpm[[y]]$JGeneName==J[x] & pt8rpm[[y]]$
           cdr3Length==cdr3L[z] & pt8rpm[[y]]$VGeneName==V[v] & pt8rpm[[y]]$
           DGeneName==d[w])]),
```

```
1104     sum(pt9rpm[[y]]$RPM[which(pt9rpm[[y]]$JGeneName==J[x] & pt9rpm[[y]]$
             cdr3Length==cdr3L[z] & pt9rpm[[y]]$VGeneName==V[v] & pt9rpm[[y]]$
             DGeneName==d[w])]))
1105     }
1106
1107  #puts the values for each subject into a matrix
1108  rindiv<-matrix(ncol=13,nrow=L)
1109  for (i in 1:L) {
1110  rindiv[i,]<-indiv(i)
1111  }
1112
1113  #print(rindiv)
1114
1115 #set 0 values to a minimum detection threshold
1116 for (m in 1:L){
1117 rindiv[m,which(rindiv[m,]==0)]<-1e-6
1118 }
1119
1120 for (m in 1:L){
1121 rgroup[m,which(rgroup[m,]==0)]<-1e-6
1122 }
1123
1124 for (m in 1:L){
1125 rbar[which(rbar==0)]<-1e-6
1126 }
1127
1128  #Calculates the between group and within group divergence
```

```
1129 bgd<-abs(sum(rgroup*log(rgroup/cbind(rbar,rbar))))
1130 #print(bgd)
1131 wgd<-sum(sum(rindiv[,c(2,5,11,7,8)]*log(rindiv[,c(2,5,11,7,8)]/cbind(rgroup
        [,1],rgroup[,1],rgroup[,1],rgroup[,1],rgroup[,1])),
1132 rindiv[,c(-2,-5,-11,-7,-8)]*log(rindiv[,c(-2,-5,-11,-7,-8)]/cbind(rgroup
        [,2],rgroup[,2],rgroup[,2],rgroup[,2],rgroup[,2],rgroup[,2],rgroup[,2],
        rgroup[,2])),na.rm=TRUE),na.rm=TRUE)
1133 #print(wgd)
1134
1135 ndot=13
1136 #uses all the parts to run the F-test
1137 Fstat<-((ndot-G+1)*bgd)/((G-1)*wgd)
1138
1139 Ftest<-Fstat/(qf(c(.025,.975), (L-1)*(G-1), (L-1)*(ndot-G+1)))
1140
1141 pval<- pf(c(.05), (L-1)*(G-1), (L-1)*(ndot-G+1))
1142
1143 list(Fstat,Ftest,pval)
1144 }
1145 }
1146
1147 VDJdist<-matrix(nrow=(length(J)*length(V)*length(d)),ncol=2)
1148 colnames(VDJdist)<-c("Fstat","pval")
1149
1150
1151 #list of VDJ combinations
1152 location<-expand.grid(J,V,d)
```

```
1153
1154 # a is VDJ combination
1155 #runs KL divergence test for each VDJ combination
1156 for (a in 1:1248){
1157 VDJKL<- VDJKLtest(which(J==location[a,1]),which(V==location[a,2]),which(d==
        location[a,3]),2)
1158 VDJdist[a,1]<-VDJKL[[1]]
1159 VDJdist[a,2]<-VDJKL[[3]]
1160 print(a)
1161 }
1162
1163 setwd("C:/Users/makowski/Desktop/Archer Code and Files")
1164 save(VDJdist,location,file="KLtest.RData")
1165
1166 #save(VDJdist,file="VDJKLtest.RData")
1167 #remove missing values
1168 load("VDJNAs.RData")
1169 rownames(VDJdist)<-c(1:1248)
1170 #run the FDR correction on pvalues
1171 results.dist<-p.adjust(as.numeric(VDJdist[-NAs,2]), method="BH")
1172 results.dist<-data.frame(pval=results.dist,index=c(1:(1248-length(NAs))))
1173 results.dist<-results.dist[order(as.numeric(VDJdist[-NAs,2])),]
1174
1175
1176
1177
1178
```

```
1179 ########################################################
1180 #This section calculates the non-parametric test
1181 ########################################################
1182
1183 setwd("C:/Users/makowski/Desktop/Archer Code and Files")
1184 load("TcellRPM.RData")
1185
1186 #Function that does the non-parametric test for the VDJ families x is the J
       gene, w is V gene and z is d gene and y=1 is donor and y=2 is recipient
1187 alphatest<-function(x,w,z,y){
1188 #this part calculates the number of unique CDR3 lengths
1189 L=length(unique(c(unique(pt11rpm[[y]]$cdr3Length[which(pt11rpm[[y]]$
       JGeneName==J[x] & pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName
       ==d[z])]),
1190         unique(pt12rpm[[y]]$cdr3Length[which(pt12rpm[[y]]$JGeneName==J[x] &
                pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z])]),
1191         unique(pt13rpm[[y]]$cdr3Length[which(pt13rpm[[y]]$JGeneName==J[x] &
                pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z])]),
1192         unique(pt14rpm[[y]]$cdr3Length[which(pt14rpm[[y]]$JGeneName==J[x] &
                pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z])]),
1193         unique(pt16rpm[[y]]$cdr3Length[which(pt16rpm[[y]]$JGeneName==J[x]&
               pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z])]),
1194         unique(pt24rpm[[y]]$cdr3Length[which(pt24rpm[[y]]$JGeneName==J[x]&
               pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z])]),
1195         unique(pt3rpm[[y]]$cdr3Length[which(pt3rpm[[y]]$JGeneName==J[x] &
               pt3rpm[[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z])]),
```

```
1196        unique(pt4rpm[[y]]$cdr3Length[which(pt4rpm[[y]]$JGeneName==J[x] &
               pt4rpm[[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z])]),
1197        unique(pt5rpm[[y]]$cdr3Length[which(pt5rpm[[y]]$JGeneName==J[x] &
               pt5rpm[[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z])]),
1198        unique(pt6rpm[[y]]$cdr3Length[which(pt6rpm[[y]]$JGeneName==J[x] &
               pt6rpm[[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z])]),
1199        unique(pt7rpm[[y]]$cdr3Length[which(pt7rpm[[y]]$JGeneName==J[x] &
               pt7rpm[[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z])]),
1200        unique(pt8rpm[[y]]$cdr3Length[which(pt8rpm[[y]]$JGeneName==J[x] &
               pt8rpm[[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z])]),
1201        unique(pt9rpm[[y]]$cdr3Length[which(pt9rpm[[y]]$JGeneName==J[x] &
               pt9rpm[[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z])])))
1202 #print(L)
1203 #keeps only VDJ combinations that have at least 2 non-zero peaks
1204 if (L<2){
1205 alphai<-rep(0,13)
1206 alphai
1207 }
1208
1209 else {
1210 #list present peaks
1211 Peaks=unique(c(unique(pt11rpm[[y]]$cdr3Length[which(pt11rpm[[y]]$JGeneName
       ==J[x] & pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName==d[z])])
       ,
1212        unique(pt12rpm[[y]]$cdr3Length[which(pt12rpm[[y]]$JGeneName==J[x] &
               pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z])]),
```

```
1213        unique(pt13rpm[[y]]$cdr3Length[which(pt13rpm[[y]]$JGeneName==J[x] &
                pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z])]),
1214        unique(pt14rpm[[y]]$cdr3Length[which(pt14rpm[[y]]$JGeneName==J[x] &
                pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z])]),
1215        unique(pt16rpm[[y]]$cdr3Length[which(pt16rpm[[y]]$JGeneName==J[x]&
                pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z])]),
1216        unique(pt24rpm[[y]]$cdr3Length[which(pt24rpm[[y]]$JGeneName==J[x]&
                pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z])]),
1217        unique(pt3rpm[[y]]$cdr3Length[which(pt3rpm[[y]]$JGeneName==J[x] &
                pt3rpm[[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z])]),
1218        unique(pt4rpm[[y]]$cdr3Length[which(pt4rpm[[y]]$JGeneName==J[x] &
                pt4rpm[[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z])]),
1219        unique(pt5rpm[[y]]$cdr3Length[which(pt5rpm[[y]]$JGeneName==J[x] &
                pt5rpm[[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z])]),
1220        unique(pt6rpm[[y]]$cdr3Length[which(pt6rpm[[y]]$JGeneName==J[x] &
                pt6rpm[[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z])]),
1221        unique(pt7rpm[[y]]$cdr3Length[which(pt7rpm[[y]]$JGeneName==J[x] &
                pt7rpm[[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z])]),
1222        unique(pt8rpm[[y]]$cdr3Length[which(pt8rpm[[y]]$JGeneName==J[x] &
                pt8rpm[[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z])]),
1223        unique(pt9rpm[[y]]$cdr3Length[which(pt9rpm[[y]]$JGeneName==J[x] &
                pt9rpm[[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z])])))
1224 #print(Peaks)
1225 #calculates total rpm per peak
1226 Peaksi<-matrix(ncol=13,nrow=L)
1227 for (i in 1:L){
```

```
1228 Peaksi[i,]<-c(sum(pt11rpm[[y]]$RPM[which(pt11rpm[[y]]$JGeneName==J[x] &
       pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName==d[z] & pt11rpm[[y
       ]]$cdr3Length==Peaks[i])]),
1229             sum(pt12rpm[[y]]$RPM[which(pt12rpm[[y]]$JGeneName==J[x] &
                    pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z]
                    & pt12rpm[[y]]$cdr3Length==Peaks[i])]),
1230             sum(pt13rpm[[y]]$RPM[which(pt13rpm[[y]]$JGeneName==J[x] &
                    pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z]
                    & pt13rpm[[y]]$cdr3Length==Peaks[i])]),
1231             sum(pt14rpm[[y]]$RPM[which(pt14rpm[[y]]$JGeneName==J[x] &
                    pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z]
                    & pt14rpm[[y]]$cdr3Length==Peaks[i])]),
1232             sum(pt16rpm[[y]]$RPM[which(pt16rpm[[y]]$JGeneName==J[x] &
                    pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z]
                    & pt16rpm[[y]]$cdr3Length==Peaks[i])]),
1233             sum(pt24rpm[[y]]$RPM[which(pt24rpm[[y]]$JGeneName==J[x] &
                    pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z]
                    & pt24rpm[[y]]$cdr3Length==Peaks[i])]),
1234             sum(pt3rpm[[y]]$RPM[which(pt3rpm[[y]]$JGeneName==J[x] & pt3rpm
                    [[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z] & pt3rpm
                    [[y]]$cdr3Length==Peaks[i])]),
1235             sum(pt4rpm[[y]]$RPM[which(pt4rpm[[y]]$JGeneName==J[x] & pt4rpm
                    [[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z] & pt4rpm
                    [[y]]$cdr3Length==Peaks[i])]),
1236             sum(pt5rpm[[y]]$RPM[which(pt5rpm[[y]]$JGeneName==J[x] & pt5rpm
                    [[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z] & pt5rpm
                    [[y]]$cdr3Length==Peaks[i])]),
```

```
1237              sum(pt6rpm[[y]]$RPM[which(pt6rpm[[y]]$JGeneName==J[x] & pt6rpm
                      [[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z] & pt6rpm
                      [[y]]$cdr3Length==Peaks[i])]),
1238              sum(pt7rpm[[y]]$RPM[which(pt7rpm[[y]]$JGeneName==J[x] & pt7rpm
                      [[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z] & pt7rpm
                      [[y]]$cdr3Length==Peaks[i])]),
1239              sum(pt8rpm[[y]]$RPM[which(pt8rpm[[y]]$JGeneName==J[x] & pt8rpm
                      [[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z] & pt8rpm
                      [[y]]$cdr3Length==Peaks[i])]),
1240              sum(pt9rpm[[y]]$RPM[which(pt9rpm[[y]]$JGeneName==J[x] & pt9rpm
                      [[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z] & pt9rpm
                      [[y]]$cdr3Length==Peaks[i])]))
1241              }
1242 #print(Peaksi)
1243 #calculates total rpm for patient with VDJ combination
1244 Peakssum<-apply(Peaksi,2,sum)
1245
1246 #print(Peakssum)
1247 #calculates probability of each peak
1248 Probabilityi<-matrix(ncol=13,nrow=L)
1249 for (j in 1:L) {
1250 Probabilityi[j,]<-Peaksi[j,]/Peakssum
1251 }
1252 #set NaN to zero
1253 for (m in 1:L){
1254 Probabilityi[m,which(Probabilityi[m,]=="NaN")]<-0
1255 }
```

```
1256
1257 #print(Probabilityi)
1258
1259 #calculate variance of each peak
1260 sigma<-sqrt(Probabilityi*(1-Probabilityi))
1261 #print(sigma)
1262 #set p for linear model
1263 p<-matrix(rep(c(0.1,0.2,0.3,0.4,0.5,0.6,0.7,0.8,0.9),1),nrow=9,ncol=1)
1264
1265 cdf<-matrix(nrow=9,ncol=13)
1266 #calculate cdf for each peak
1267 for(k in 1:13){
1268    for(o in 1:9){
1269 cdf[o,k]<-(1/L)*sum(pnorm(p[o]-Probabilityi[,k],0,sigma[,k]))
1270   }
1271   }
1272
1273
1274 #print(cdf)
1275
1276 alphai<-matrix(nrow=1,ncol=13)
1277 #calculate alpha value using lm
1278 for(l in 1:13){
1279 alpha<-lm(log10(cdf[,l])~log10(p))
1280 alphai[l]<-alpha$coef[1]
1281 }
1282 #print(alphai)
```

```
1283
1284
1285 #plot(log10(p),log10(cdf[,1]))
1286
1287 alphai
1288 }
1289 }
1290 #list of VDJ combinations
1291 location<-expand.grid(J,V,d)
1292
1293 # a is VDJ combination
1294 #calculates alpha values for each VDJ combination
1295 alphapower<-matrix(nrow=(length(J)*length(V)*length(d)),ncol=13)
1296 for (a in 1:1248){
1297 alphapower[a,]<-alphatest(which(J==location[a,1]),which(V==location[a,2]),
       which(d==location[a,3]),2)
1298 print(a)
1299 }
1300
1301 setwd("C:/Users/makowski/Desktop/Archer Code and Files")
1302 save(alphapower,location,file="AlphaTest.RData")
1303
1304
1305
1306
1307
1308
```

```
1309 ##############################################
1310 #Run the permutation tests for group differences
1311 ##############################################
1312
1313
1314 GroupGVT<-c(2,5,7,8,11)
1315 GroupGVHD<-c(1,3,4,6,9,10,12,13)
1316
1317 #GVT is 0 and GVHD is 1
1318 Group<-c(1,0,1,1,0,1,0,0,1,1,0,1,1)
1319
1320 #for referencing which VDJ combination
1321 rownames(alphapower)<-c(1:1248)
1322 #load NA values for tests
1323 setwd("C:/Users/makowski/Desktop/Archer Code and Files")
1324 load("VDJNAs.RData")
1325
1326 alphapower.NA<-alphapower[-NAs,]
1327
1328 set.seed(123)
1329 B <- 1000
1330 test.stat<-pval<-numeric()
1331 test.stat.b<-matrix(nrow=dim(alphapower.NA)[1], ncol=B)
1332 for (i in 1:dim(alphapower.NA)[1]) {
1333
1334         test.stat[i]<-mean(alphapower.NA[i,Group==1],na.rm=TRUE) - mean(
                alphapower.NA[i,Group==0],na.rm=TRUE)
```

```
1335         for (j in 1:B) {
1336
1337                sample<-sample(Group, replace=FALSE)
1338                 test.stat.b[i,j]<-mean(alphapower.NA[i,sample==1],na.rm=TRUE
                        ) - mean(alphapower.NA[i,sample==0],na.rm=TRUE)
1339         }
1340         pval[i]<-sum(abs(test.stat.b[i,]>abs(test.stat[i])))/B
1341 }
1342
1343 fdr<-cbind(p.adjust(pval, method="BH"),c(1:dim(alphapower.NA)[1]))
1344 length(which(fdr[,1]<0.05))
1345 fdr.order<-fdr[order(pval),]
1346 location.NA<-location[-NAs,]
1347
1348 groupnames<-factor(Group,levels=c(0,1),labels=c("No GVHD","GVHD"))
1349 #make some plots
1350 png(file = "nonparametrictest.png", width = 1200, height = 1000, units = "px
        ")
1351 par(mfrow=c(1,1),mar=c(5.1,4.5,4.1,2.1))
1352 plot(groupnames,alphapower.NA[fdr.order[1,2],],main=paste(location.NA[fdr.
        order[1,2],1],location.NA[fdr.order[1,2],2],location.NA[fdr.order
        [1,2],3]),cex.lab=2,cex.main=2,cex.axis=2,ylab="Alpha")
1353 dev.off()
1354
1355
1356 library(xtable)
```

```
apply(t(alphapower.NA[fdr.order[c(1:1),2],GroupGVT]),2,FUN=median,na.rm=TRUE
    )
perttest.table<-cbind(location.NA[fdr.order[c(1:1),2],],median(alphapower.NA
    [fdr.order[c(1:1),2],GroupGVT],na.rm=TRUE),median(alphapower.NA[fdr.
    order[c(1:1),2],GroupGVHD],na.rm=TRUE),fdr.order[c(1:1),1])
colnames(perttest.table)<-c("JGene","VGene","DGene", "Median No GVHD
    Perturbation", "Median GVHD Perturbation", "FDR")
xtable(perttest.table,digits=3)

############################################################
#This section runs the logit transformation
############################################################

setwd("C:/Users/makowski/Desktop/Archer Code and Files")
load("TcellRPM.RData")

#function to pull out unique and shared counts
countrpm<-function(patient){
counta<-matrix(nrow=624,ncol=5)
for (i in 1:48) {
for (j in 1:13) {
for (d in 2:2) {
pt3d.VJ<-subset(patient[[1]],VGeneName==V[i] & JGeneName==J[j] & DGeneName==
    Dg[d])
pt3r.VJ<-subset(patient[[2]],VGeneName==V[i] & JGeneName==J[j] & DGeneName==
    Dg[d])
```

```
1377 pt3<-merge(pt3d.VJ,pt3r.VJ,by.x="aminoAcid",by.y="aminoAcid",all.x=TRUE,all.
        y=TRUE)
1378 unique.3<-sum(pt3$RPM.y[which(!is.na(pt3$RPM.y)=="TRUE" & !is.na(pt3$RPM.x)
        =="FALSE")])
1379 shared.3<-sum(pt3$RPM.y[which(!is.na(pt3$RPM.y)=="TRUE" & !is.na(pt3$RPM.x)
        =="TRUE")])
1380 counta[((i-1)*13)+j,]<-cbind(V[i],J[j],Dg[d],unique.3,shared.3)
1381 }
1382 }
1383 }
1384
1385 countb<-matrix(nrow=624,ncol=5)
1386 for (i in 1:48) {
1387 for (j in 1:13) {
1388 for (d in 3:3) {
1389 pt3d.VJ<-subset(patient[[1]],VGeneName==V[i] & JGeneName==J[j] & DGeneName==
        Dg[d])
1390 pt3r.VJ<-subset(patient[[2]],VGeneName==V[i] & JGeneName==J[j] & DGeneName==
        Dg[d])
1391 pt3<-merge(pt3d.VJ,pt3r.VJ,by.x="aminoAcid",by.y="aminoAcid",all.x=TRUE,all.
        y=TRUE)
1392 unique.3<-sum(pt3$RPM.y[which(!is.na(pt3$RPM.y)=="TRUE" & !is.na(pt3$RPM.x)
        =="FALSE")])
1393 shared.3<-sum(pt3$RPM.y[which(!is.na(pt3$RPM.y)=="TRUE" & !is.na(pt3$RPM.x)
        =="TRUE")])
1394 countb[((i-1)*13)+j,]<-cbind(V[i],J[j],Dg[d],unique.3,shared.3)
1395 }
```

```
}
}

count<- rbind(counta,countb)
colnames(count)<-c("VGene","JGene","Dgene","Unique","Shared")
list(count)
}


count3<-countrpm(pt3rpm)
count4<-countrpm(pt4rpm)
count5<-countrpm(pt5rpm)
count6<-countrpm(pt6rpm)
count7<-countrpm(pt7rpm)
count8<-countrpm(pt8rpm)
count9<-countrpm(pt9rpm)
count11<-countrpm(pt11rpm)
count12<-countrpm(pt12rpm)
count13<-countrpm(pt13rpm)
count14<-countrpm(pt14rpm)
count16<-countrpm(pt16rpm)
count24<-countrpm(pt24rpm)

save(count3,count4,count5,count6,count7,count8,count9,count11,count12,
    count13,count14,count16,count24,file="TcellCount.RData")

#function to do calculate proportion and logit transformation
```

```
1422 fam2<-function(fam){
1423 fam<-cbind(fam[[1]],as.numeric(fam[[1]][,4])/(as.numeric(fam[[1]][,4])+as.
        numeric(fam[[1]][,5])))
1424 colnames(fam)[6]<-"proportion"
1425 fam<-cbind(fam,as.numeric(fam[,6])/(1-as.numeric(fam[,6])))
1426 colnames(fam)[7]<-"transform"
1427 return(fam)
1428 }
1429
1430 fam11<-fam2(count11)
1431 fam12<-fam2(count12)
1432 fam13<-fam2(count13)
1433 fam14<-fam2(count14)
1434 fam16<-fam2(count16)
1435 fam24<-fam2(count24)
1436 fam3<-fam2(count3)
1437 fam4<-fam2(count4)
1438 fam5<-fam2(count5)
1439 fam6<-fam2(count6)
1440 fam7<-fam2(count7)
1441 fam8<-fam2(count8)
1442 fam9<-fam2(count9)
1443
1444 famVDJ<-cbind(as.numeric(fam11[,7]),
1445              as.numeric(fam12[,7]),
1446              as.numeric(fam13[,7]),
1447              as.numeric(fam14[,7]),
```

```
1448              as.numeric(fam16[,7]),
1449              as.numeric(fam24[,7]),
1450              as.numeric(fam3[,7]),
1451              as.numeric(fam4[,7]),
1452              as.numeric(fam5[,7]),
1453              as.numeric(fam6[,7]),
1454              as.numeric(fam7[,7]),
1455              as.numeric(fam8[,7]),
1456              as.numeric(fam9[,7]))
1457
1458 colnames(famVDJ)<-cbind("fam11$transform",
1459              "fam12$transform",
1460              "fam13$transform",
1461              "fam14$transform",
1462              "fam16$transform",
1463              "fam24$transform",
1464              "fam3$transform",
1465              "fam4$transform",
1466              "fam5$transform",
1467              "fam6$transform",
1468              "fam7$transform",
1469              "fam8$transform",
1470              "fam9$transform")
1471
1472
1473 #replace inf and NaN with 0
1474 for (i in 1:1248){
```

```
   for (j in 1:13){
   if (is.nan(famVDJ[i,j])=="TRUE"){
famVDJ[i,j]<-0
                     }
   else if (famVDJ[i,j]=="Inf"){
famVDJ[i,j]<-0
                     }
              }
               }

#Reorder rows to match other analyses
load("VDJobjects.RData")
location<-expand.grid(J,V,d)

order.logit<-matrix(nrow=1248,ncol=1)
for (i in 1:1248) {
order.logit[i]<-which(location[,1]==count3[[1]][i,2] & location[,2]==count3
    [[1]][i,1] & location[,3]==count3[[1]][i,3])
}

#for referencing which VDJ combination
rownames(famVDJ)<-c(1:1248)
#load NA values for tests
setwd("C:/Users/makowski/Desktop/Archer Code and Files")
load("VDJNAs.RData")

famVDJ.NAs<-famVDJ[-NAs,]
```

```
1501
1502 #group indicator
1503 group<-c("GVHD","GVT","GVHD","GVHD","GVT","GVHD","GVT","GVT","GVHD","GVHD","
        GVT","GVHD","GVHD")
1504 #run linear model to test differences
1505 res.lm<-matrix(nrow=1248-length(NAs),ncol=2)
1506 for (i in 1:(1248-length(NAs))){
1507 prop.lm<-lm(famVDJ.NAs[i,]~as.factor(group))
1508 pv.lm<-summary(prop.lm)$coef[2, "Pr(>|t|)"]
1509 cv.lm<-coef(prop.lm)[2]
1510 res.lm[i,]<-cbind(pv.lm,cv.lm)
1511 }
1512
1513 colnames(res.lm)<-c("pvalue","coefficient")
1514
1515 fdr<-cbind(p.adjust(res.lm[,1], method="BH"),c(1:dim(famVDJ.NAs)[1]))
1516 length(which(fdr[,1]<0.05))
1517 fdr.order<-fdr[order(res.lm[,1]),]
1518 location.NA<-location[-NAs,]
1519 #GVT is 0 and GVHD is 1
1520 Group<-c(1,0,1,1,0,1,0,0,1,1,0,1,1)
1521 groupnames<-factor(Group,levels=c(0,1),labels=c("No GVHD","GVHD"))
1522 #make some plots
1523 png(file = "famVDJ.png", width = 1200, height = 1000, units = "px")
1524 par(mfrow=c(1,2),mar=c(5.1,4.5,4.1,2.1))
1525 plot(groupnames,famVDJ.NAs[fdr.order[1,2],],main=paste(location.NA[fdr.order
        [1,2],1],location.NA[fdr.order[1,2],2],location.NA[fdr.order[1,2],3]),
```

```
      cex.lab=2,cex.main=2,cex.axis=2,ylab="Logit Proportion Shared")
1526 plot(groupnames,famVDJ.NAs[fdr.order[2,2],],main=paste(location.NA[fdr.order
      [2,2],1],location.NA[fdr.order[2,2],2],location.NA[fdr.order[2,2],3]),
      cex.lab=2,cex.main=2,cex.axis=2,ylab="Logit Proportion Shared")
1527 dev.off()
1528
1529 GroupGVT<-c(2,5,7,8,11)
1530 GroupGVHD<-c(1,3,4,6,9,10,12,13)
1531
1532 library(xtable)
1533 apply(t(famVDJ.NAs[fdr.order[c(1:2),2],GroupGVT]),2,FUN=median,na.rm=TRUE)
1534 perttest.table<-cbind(location.NA[fdr.order[c(1:2),2],],apply(t(famVDJ.NAs[
      fdr.order[c(1:2),2],GroupGVT]),2,FUN=median,na.rm=TRUE),apply(t(famVDJ.
      NAs[fdr.order[c(1:2),2],GroupGVHD]),2,FUN=median,na.rm=TRUE),res.lm[fdr.
      order[c(1:2),2],1])
1535 colnames(perttest.table)<-c("JGene","VGene","DGene", "Median No GVHD
      Perturbation", "Median GVHD Perturbation", "FDR")
1536 xtable(perttest.table,digits=3)
```

6.2 Compositional Code

```
1
2 setwd("C:/Users/makowski/Desktop/Archer Code and Files")
3 load("TcellRPM.RData")
4 load("VDJobjects.RData")
5 library(compositions)
6
7 #Sum all VDJ combinations
8 location<-expand.grid(J,V,d)
9 VDJcomp<-matrix(ncol=1248,nrow=13)
10 for (i in 1:1248){
11 y<-2
12 x<-which(J==location[i,1])
13 w<-which(V==location[i,2])
14 z<-which(d==location[i,3])
15 VDJcomp[,i]<-c(sum(pt11rpm[[y]]$RPM[which(pt11rpm[[y]]$JGeneName==J[x] &
     pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName==d[z])]),
16              sum(pt12rpm[[y]]$RPM[which(pt12rpm[[y]]$JGeneName==J[x] &
                    pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z])
                    ]),
17              sum(pt13rpm[[y]]$RPM[which(pt13rpm[[y]]$JGeneName==J[x] &
                    pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z])
                    ]),
18              sum(pt14rpm[[y]]$RPM[which(pt14rpm[[y]]$JGeneName==J[x] &
                    pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z])
                    ]),
```

```
19              sum(pt16rpm[[y]]$RPM[which(pt16rpm[[y]]$JGeneName==J[x] &
                   pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z])
                   ]),
20              sum(pt24rpm[[y]]$RPM[which(pt24rpm[[y]]$JGeneName==J[x] &
                   pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z])
                   ]),
21              sum(pt3rpm[[y]]$RPM[which(pt3rpm[[y]]$JGeneName==J[x] & pt3rpm
                   [[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z])]),
22              sum(pt4rpm[[y]]$RPM[which(pt4rpm[[y]]$JGeneName==J[x] & pt4rpm
                   [[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z])]),
23              sum(pt5rpm[[y]]$RPM[which(pt5rpm[[y]]$JGeneName==J[x] & pt5rpm
                   [[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z])]),
24              sum(pt6rpm[[y]]$RPM[which(pt6rpm[[y]]$JGeneName==J[x] & pt6rpm
                   [[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z])]),
25              sum(pt7rpm[[y]]$RPM[which(pt7rpm[[y]]$JGeneName==J[x] & pt7rpm
                   [[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z])]),
26              sum(pt8rpm[[y]]$RPM[which(pt8rpm[[y]]$JGeneName==J[x] & pt8rpm
                   [[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z])]),
27              sum(pt9rpm[[y]]$RPM[which(pt9rpm[[y]]$JGeneName==J[x] & pt9rpm
                   [[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z])]))
28              }
29
30 #check to see where 0's are
31 Diversity.check<-matrix(rep(0,(1248*13)),ncol=1248,nrow=13)
32 for (i in 1:13){
33   for (j in 1:1248){
34
```

```
if (VDJcomp[i,j]=="0"){
Diversity.check[i,j]<-1
                    }
    else {
Diversity.check[i,j]<-0
                    }
              }
               }

#keep only VDJ combinations that 7 or more of the sample contain
NAs<-c(which(apply(Diversity.check,2,sum)==13),which(apply(Diversity.check
    ,2,sum)==12),which(apply(Diversity.check,2,sum)==11),which(apply(
    Diversity.check,2,sum)==10),which(apply(Diversity.check,2,sum)==9),which
    (apply(Diversity.check,2,sum)==8),which(apply(Diversity.check,2,sum)==7)
    ,which(apply(Diversity.check,2,sum)==6))

#save(NAs,file="VDJNAs.RData")

VDJcomp.NA<-VDJcomp[,-NAs]

#set zeros to 1e-6
for (i in 1:13){
   for (j in 1:(1248-length(NAs))){
if (VDJcomp.NA[i,j]=="0")
VDJcomp.NA[i,j]<-1e-6
              }
               }
```

```
58
59 #top 10% most variable VDJ combinations
60 var.top<-order(apply(VDJcomp.NA,2,var))[852:947]
61
62 #tell package we are working with compositional data
63 VDJcomp.NA.a<-acomp(VDJcomp.NA)
64 rownames(VDJcomp.NA.a)<-groupnames
65 VDJcomp.mean<-mean(VDJcomp.NA.a)
66
67 #run clustering on the top 10% most variable VDJ combinations
68 Group<-c(1,0,1,1,0,1,0,0,1,1,0,1,1)
69 groupnames<-factor(Group,levels=c(0,1),labels=c("No GVHD","GVHD"))
70 dd<-dist(VDJcomp.NA.a[,var.top])
71 hc = hclust(dd, method="ward.D")
72
73 png(file = 'compcluster.png', width = 1200, height = 1000, units = "px")
74 plot(hc,xlab="Group",ylab="Aitchison Distance",main="")
75 dev.off()
76
77 save.image("compositions.RData")
78
79 ##########################################################
80
81 #Function that does the composition test for the VDJ families; x is the J
      gene, w is V gene and z is d gene and y=1 is donor and y=2 is recipient
82 prob.calc<-function(x,w,z,y){
83 #this part calculates the number of unique CDR3 lengths
```

```
84 L=length(unique(c(unique(pt11rpm[[y]]$cdr3Length[which(pt11rpm[[y]]$
     JGeneName==J[x] & pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName
     ==d[z])]),
85         unique(pt12rpm[[y]]$cdr3Length[which(pt12rpm[[y]]$JGeneName==J[x] &
               pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z])]),
86         unique(pt13rpm[[y]]$cdr3Length[which(pt13rpm[[y]]$JGeneName==J[x] &
               pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z])]),
87         unique(pt14rpm[[y]]$cdr3Length[which(pt14rpm[[y]]$JGeneName==J[x] &
               pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z])]),
88         unique(pt16rpm[[y]]$cdr3Length[which(pt16rpm[[y]]$JGeneName==J[x]&
              pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z])]),
89         unique(pt24rpm[[y]]$cdr3Length[which(pt24rpm[[y]]$JGeneName==J[x]&
              pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z])]),
90         unique(pt3rpm[[y]]$cdr3Length[which(pt3rpm[[y]]$JGeneName==J[x] &
              pt3rpm[[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z])]),
91         unique(pt4rpm[[y]]$cdr3Length[which(pt4rpm[[y]]$JGeneName==J[x] &
              pt4rpm[[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z])]),
92         unique(pt5rpm[[y]]$cdr3Length[which(pt5rpm[[y]]$JGeneName==J[x] &
              pt5rpm[[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z])]),
93         unique(pt6rpm[[y]]$cdr3Length[which(pt6rpm[[y]]$JGeneName==J[x] &
              pt6rpm[[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z])]),
94         unique(pt7rpm[[y]]$cdr3Length[which(pt7rpm[[y]]$JGeneName==J[x] &
              pt7rpm[[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z])]),
95         unique(pt8rpm[[y]]$cdr3Length[which(pt8rpm[[y]]$JGeneName==J[x] &
              pt8rpm[[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z])]),
96         unique(pt9rpm[[y]]$cdr3Length[which(pt9rpm[[y]]$JGeneName==J[x] &
              pt9rpm[[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z])])))
```

```
97 #print(L)
98
99 #this sets the perturbation statistic to zero if the VDJ combination is not
       present
100 if (L==0){
101 AbsoluteDeviancei<-rep(0,13)
102 AbsoluteDeviancei
103 }
104
105 else {
106 #This list the peak lengths
107 Peaks=unique(c(unique(pt11rpm[[y]]$cdr3Length[which(pt11rpm[[y]]$JGeneName
      ==J[x] & pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName==d[z])])
      ,
108         unique(pt12rpm[[y]]$cdr3Length[which(pt12rpm[[y]]$JGeneName==J[x] &
              pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z])]),
109         unique(pt13rpm[[y]]$cdr3Length[which(pt13rpm[[y]]$JGeneName==J[x] &
              pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z])]),
110         unique(pt14rpm[[y]]$cdr3Length[which(pt14rpm[[y]]$JGeneName==J[x] &
              pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z])]),
111         unique(pt16rpm[[y]]$cdr3Length[which(pt16rpm[[y]]$JGeneName==J[x]&
             pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z])]),
112         unique(pt24rpm[[y]]$cdr3Length[which(pt24rpm[[y]]$JGeneName==J[x]&
             pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z])]),
113         unique(pt3rpm[[y]]$cdr3Length[which(pt3rpm[[y]]$JGeneName==J[x] &
             pt3rpm[[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z])]),
```

```
114         unique(pt4rpm[[y]]$cdr3Length[which(pt4rpm[[y]]$JGeneName==J[x] &
               pt4rpm[[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z])]),
115         unique(pt5rpm[[y]]$cdr3Length[which(pt5rpm[[y]]$JGeneName==J[x] &
               pt5rpm[[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z])]),
116         unique(pt6rpm[[y]]$cdr3Length[which(pt6rpm[[y]]$JGeneName==J[x] &
               pt6rpm[[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z])]),
117         unique(pt7rpm[[y]]$cdr3Length[which(pt7rpm[[y]]$JGeneName==J[x] &
               pt7rpm[[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z])]),
118         unique(pt8rpm[[y]]$cdr3Length[which(pt8rpm[[y]]$JGeneName==J[x] &
               pt8rpm[[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z])]),
119         unique(pt9rpm[[y]]$cdr3Length[which(pt9rpm[[y]]$JGeneName==J[x] &
               pt9rpm[[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z])])))
120 #print(Peaks)
121 #this calculates the total rpm in each peack
122 Peaksi<-matrix(ncol=13,nrow=L)
123 for (i in 1:L){
124 Peaksi[i,]<-c(sum(pt11rpm[[y]]$RPM[which(pt11rpm[[y]]$JGeneName==J[x] &
      pt11rpm[[y]]$VGeneName==V[w] & pt11rpm[[y]]$DGeneName==d[z] & pt11rpm[[y
      ]]$cdr3Length==Peaks[i])]),
125             sum(pt12rpm[[y]]$RPM[which(pt12rpm[[y]]$JGeneName==J[x] &
                   pt12rpm[[y]]$VGeneName==V[w] & pt12rpm[[y]]$DGeneName==d[z]
                   & pt12rpm[[y]]$cdr3Length==Peaks[i])]),
126             sum(pt13rpm[[y]]$RPM[which(pt13rpm[[y]]$JGeneName==J[x] &
                   pt13rpm[[y]]$VGeneName==V[w] & pt13rpm[[y]]$DGeneName==d[z]
                   & pt13rpm[[y]]$cdr3Length==Peaks[i])]),
127             sum(pt14rpm[[y]]$RPM[which(pt14rpm[[y]]$JGeneName==J[x] &
                   pt14rpm[[y]]$VGeneName==V[w] & pt14rpm[[y]]$DGeneName==d[z]
```

```
    & pt14rpm[[y]]$cdr3Length==Peaks[i])]),
128 sum(pt16rpm[[y]]$RPM[which(pt16rpm[[y]]$JGeneName==J[x] &
    pt16rpm[[y]]$VGeneName==V[w] & pt16rpm[[y]]$DGeneName==d[z]
    & pt16rpm[[y]]$cdr3Length==Peaks[i])]),
129 sum(pt24rpm[[y]]$RPM[which(pt24rpm[[y]]$JGeneName==J[x] &
    pt24rpm[[y]]$VGeneName==V[w] & pt24rpm[[y]]$DGeneName==d[z]
    & pt24rpm[[y]]$cdr3Length==Peaks[i])]),
130 sum(pt3rpm[[y]]$RPM[which(pt3rpm[[y]]$JGeneName==J[x] & pt3rpm
    [[y]]$VGeneName==V[w] & pt3rpm[[y]]$DGeneName==d[z] & pt3rpm
    [[y]]$cdr3Length==Peaks[i])]),
131 sum(pt4rpm[[y]]$RPM[which(pt4rpm[[y]]$JGeneName==J[x] & pt4rpm
    [[y]]$VGeneName==V[w] & pt4rpm[[y]]$DGeneName==d[z] & pt4rpm
    [[y]]$cdr3Length==Peaks[i])]),
132 sum(pt5rpm[[y]]$RPM[which(pt5rpm[[y]]$JGeneName==J[x] & pt5rpm
    [[y]]$VGeneName==V[w] & pt5rpm[[y]]$DGeneName==d[z] & pt5rpm
    [[y]]$cdr3Length==Peaks[i])]),
133 sum(pt6rpm[[y]]$RPM[which(pt6rpm[[y]]$JGeneName==J[x] & pt6rpm
    [[y]]$VGeneName==V[w] & pt6rpm[[y]]$DGeneName==d[z] & pt6rpm
    [[y]]$cdr3Length==Peaks[i])]),
134 sum(pt7rpm[[y]]$RPM[which(pt7rpm[[y]]$JGeneName==J[x] & pt7rpm
    [[y]]$VGeneName==V[w] & pt7rpm[[y]]$DGeneName==d[z] & pt7rpm
    [[y]]$cdr3Length==Peaks[i])]),
135 sum(pt8rpm[[y]]$RPM[which(pt8rpm[[y]]$JGeneName==J[x] & pt8rpm
    [[y]]$VGeneName==V[w] & pt8rpm[[y]]$DGeneName==d[z] & pt8rpm
    [[y]]$cdr3Length==Peaks[i])]),
136 sum(pt9rpm[[y]]$RPM[which(pt9rpm[[y]]$JGeneName==J[x] & pt9rpm
    [[y]]$VGeneName==V[w] & pt9rpm[[y]]$DGeneName==d[z] & pt9rpm
```

```
                [[y]]$cdr3Length==Peaks[i])]))
137             }
138 #print(Peaksi)
139 #total rpm per patient
140 Peakssum<-apply(Peaksi,2,sum)
141
142 #print(Peakssum)
143 #calculates the probability of each peak
144 Probabilityi<-matrix(ncol=13,nrow=L)
145 for (j in 1:L) {
146 Probabilityi[j,]<-Peaksi[j,]/Peakssum
147 }
148
149 #replace zero values with below limit of detection value 1e-6
150 for (m in 1:L){
151 Probabilityi[m,which(Probabilityi[m,]=="NaN")]<-1e-6
152 Probabilityi[m,which(Probabilityi[m,]==0)]<-1e-6
153 }
154
155 Probabilityi
156 }
157 }
158
159
160 Group<-c(1,0,1,1,0,1,0,0,1,1,0,1,1)
161 groupnames<-factor(Group,levels=c(0,1),labels=c("No GVHD","GVHD"))
162
```

```
#Run linear model for each VDJ combination
comp.pval<-matrix(nrow=1,ncol=(1248-length(NAs)))
location.NA<-location[-NAs,]
for (a in 1:947){
tryCatch({
prob.test<-prob.calc(which(J==location.NA[a,1]),which(V==location.NA[a,2]),
    which(d==location.NA[a,3]),2)
prob.test.a<-acomp(prob.test)
(model = lm(ilr(t(prob.test.a))~groupnames))
comp.pval[a]<-anova(model)[2,6]
print(a)},error=function(e){})
}

save(comp.pval,file="comppval.RData")

#identify significant values
fdr<-cbind(p.adjust(comp.pval, method="BH"),c(1:dim(comp.pval)[2]))
length(which(fdr[,1]<0.05))
fdr.order<-fdr[order(comp.pval),]
location.NA<-location[-NAs,]

#make table
GroupGVT<-c(2,5,7,8,11)
GroupGVHD<-c(1,3,4,6,9,10,12,13)
load("VDJobjects.RData")
location<-expand.grid(J,V,d)
library(xtable)
```

```
189 diversitytest.table<-cbind(location.NA[fdr.order[c(1:20),2],],comp.pval[fdr.
       order[c(1:20),2]])
190 colnames(diversitytest.table)<-c("JGene","VGene","DGene", "p-value")
191 xtable(diversitytest.table,digits=3)
```

6.3 GMIFS code

6.3.1 GMIFS and Related Functions

```
poisson.theta<-function (par, w, x, y, offset, beta) {
  if (is.null(x)) {
    if (!is.null(offset)) {
      Xb <- cbind(offset, w) %*% c(1, par)
    } else {
        Xb <- w %*% par
      }
    } else {
      if (!is.null(offset)) {
          Xb <- cbind(offset, w, x) %*% c(1, par, beta)
    } else {
        Xb <- cbind(w, x) %*% c(par, beta)
      }
  }
    contri.LL<-y*Xb-exp(Xb)-lgamma(y+1) #likelihood function
    loglik <- sum(contri.LL)
    -loglik
}

poisson.gmifs<-function (formula, data, x=NULL, offset, subset, epsilon
    =0.001, tol=1e-5, scale=TRUE, verbose=FALSE, ...) {
    mf <- match.call(expand.dots = FALSE)
    cl <- match.call()
    m <- match(c("formula", "data", "subset", "offset"), names(mf), 0L)
```

```
24    mf <- mf[c(1L, m)]
25    mf[[1L]] <- as.name("model.frame")
26    mf <- eval(mf, parent.frame())
27    mt <- attr(mf, "terms")
28    y <- model.response(mf)
29    w <- model.matrix(mt, mf)
30    offset <- model.offset(mf)
31    if (!is.null(x)) { ############### Subset code
32      if (missing(subset))
33          r <- TRUE
34      else {
35          e <- substitute( subset)
36          r <- eval( e, data)
37          if (!is.logical(r))
38              stop("'subset' must evaluate to logical" )
39          r <- r & !is.na(r)
40      }
41        if (class(x)=="character") {
42            nl <- as.list( 1:ncol(data))
43            names(nl) <- names( data)
44            vars <- eval(substitute(x), nl, parent.frame())
45          x <- data [r , vars, drop=FALSE ]
46            x <- as.matrix(x )
47        } else if (class(x)== "matrix" || class(x)== "data.frame") {
48                x <- x[r,, drop =FALSE]
49                x <- as.matrix(x)
50        }
```

```
51    } ################ End subset code
52    data <- data.matrix(data)
53    theta <- rep(0, dim(w)[2]) #initiate non-penalized coefficients
54  if (!is.null(x)) {
55    vars <- dim(x)[2]
56    oldx <- x
57    if (scale) {
58      x <- scale(x, center = TRUE, scale = TRUE)
59      }
60      x <- cbind(x, -1 * x)
61      beta <- rep(0, dim(x)[2])
62      names(beta) <- dimnames(x)[[2]]
63      theta.update <- matrix(theta, ncol = length(theta))
64      step <- 0
65      Estimates <- matrix(0,ncol=vars)
66      Likelihood <- numeric()
67      AIC <- numeric()
68        #initialize theta and (Intercept)
69      step <- 0
70 #  initialize <- nlm(poisson.theta, p=theta, w=w, x=x, y=y, offset=offset,
     beta=beta)
71 #  theta <- initialize$estimate
72    initialize<-glm(y~w-1, offset=offset, family=poisson)
73    theta <- coef(initialize)
74      theta.update <- matrix(theta, ncol = length(theta))
75      repeat {
76      if (!is.null(offset)) {
```

```
77          Xb <- cbind(offset, w, x) %*% c(1,theta,beta)
78      } else {
79        Xb <- cbind(w, x) %*% c(theta,beta)
80      }
81          u <- t(x)%*%(y-exp(Xb)) #likelihood gradient value
82
83
84        update.j <- which.min(-u) #choose coeffiecient to update
85        if (-u[update.j] < 0) {
86            beta[update.j] <- beta[update.j] + epsilon #update beta
87        }
88        Estimates<-rbind(Estimates,beta[1:vars]-beta[(vars+1):length(beta)
              ]) #keep track of beta changes
89        out <- optim(theta, poisson.theta, w=w, x=x, y=y, offset=offset,
              beta=beta,method="BFGS") #update intercept and non-penalized
              subset using new beta values
90        theta <- out$par
91        theta.update <- rbind(theta.update, theta) #keep track of new theta
              values
92        p <- sum(Estimates[step+2,]!=0) + length(theta)
93        Likelihood[step+1]<- LL1<- -out$value
94        AIC[step+1]<- 2*p-2*Likelihood[step+1]
95      if (verbose) cat("step = ", step, "\n")
96        if (step >= 1 && LL1 - LL0 < tol) { #decide when to stop repeat
97            break
98        }
99        LL0 <- LL1
```

```
100           step <- 1 + step
101       }
102       beta.final <- Estimates[-1,] #final values
103         theta<-theta.update[-1,]
104         if (dim(w)[2]==grep("(Intercept)",colnames(w))) {
105           names(theta)<-colnames(w)
106         } else {
107           colnames(theta)<-colnames(w)
108         }
109         model.select<-which.min(AIC)
110       output<-list(beta = beta.final, theta=theta, x=oldx, y=y, scale=scale,
              Likelihood=Likelihood, AIC=AIC, model.select=model.select,w=w,
             offset=offset)
111     } else {
112 #   out <- nlm(poisson.theta, p=theta, w=w, x=NULL, y=y, offset=offset, beta=
      NULL)
113 #   output<- out$estimate
114     out<-glm(y~w-1, offset=offset, family=poisson)
115     output <- coef(out)
116   }
117   class(output) <- "poisson.gmifs"
118   output
119 }
120
121
122
123
```

```
124 ##############################
125 #Predict function#########
126 ##############################
127 predict.poisson.gmifs<- function(fit, newx=NULL, newoffset=NULL, model.
       select=NA) {
128 y<-fit$y
129
130 if (is.null(newx)) {
131 x<-fit$x
132 }
133 else { x<-newx
134 }
135
136 w<-fit$w
137
138 if (is.null(newoffset)){
139 offset<-fit$offset
140 }
141 else {offset<-newoffset
142 }
143
144 if (is.numeric(model.select)) {
145 model.select = model.select
146 }
147 else if (is.na(model.select)) {
148 model.select=dim(fit$beta)[1]
149 }
```

```
150 else if (model.select=="AIC") {
151 model.select = fit$model.select
152 }
153
154
155 if (is.null(dim(fit$theta))) {
156 beta<-fit$beta[model.select,]
157 theta<-fit$theta[model.select]
158 offset<-fit$offset
159
160 if (is.null(offset)) {
161 y.pred <- exp(c(theta,beta) %*% t(cbind(w, x)))
162 }
163 else {
164     offset<-fit$offset
165     y.pred <- exp(c(1,theta,beta) %*% t(cbind(offset, w, x)))
166    }
167 }
168
169 else if (is.numeric(fit$theta[,-1])){
170 beta<-fit$beta[model.select,]
171 theta<-fit$theta[model.select,]
172 offset<-fit$offset
173
174 if (is.null(offset)) {
175 y.pred <- exp(c(theta,beta) %*% t(cbind(w, x)))
176 }
```

```
177 else {
178 y.pred <- exp(c(1,theta,beta) %*% t(cbind(offset, w, x)))
179 }
180 }
181
182 output<-list(pred=y.pred,theta=theta,beta=beta,offset=offset,w=w,x=x)
183 output
184 }
185
186
187 ##############################
188 #Coefficient function#########
189 ##############################
190 coef.poisson.gmifs<- function(fit, model.select=NA) {
191
192 if (is.numeric(model.select)) {
193
194 if (is.null(dim(fit$theta))) {
195 beta<-fit$beta[model.select,]
196 theta<-fit$theta[model.select]
197 c.coef<-cbind(theta,beta)
198 colnames(c.coef)<- c("intercept",colnames(fit$w)[-1],colnames(fit$x))
199 rownames(c.coef)<-as.character(1:dim(beta)[1])
200 }
201 else {
202 beta<-fit$beta[model.select,]
203 theta<-fit$theta[model.select,]
```

```
c.coef<-cbind(theta,beta)
colnames(c.coef)<- c("intercept",colnames(fit$w)[-1],colnames(fit$x))
rownames(c.coef)<-as.character(1:dim(beta)[1])
}
}


else if (is.na(model.select)) {
model.select=dim(fit$beta)[1]
if (is.null(dim(fit$theta))) {
beta<-fit$beta[model.select,]
theta<-fit$theta[model.select]
c.coef<-c(theta,beta)

}
else {
beta<-fit$beta[model.select,]
theta<-fit$theta[model.select,]
c.coef<-c(theta,beta)

}
}

else if (model.select == "AIC") {
      model.select = fit$model.select
if (is.null(dim(fit$theta))) {
beta<-fit$beta[model.select,]
```

```
231 theta<-fit$theta[model.select]
232 c.coef<-c(theta,beta)
233
234 }
235 else {
236 beta<-fit$beta[model.select,]
237 theta<-fit$theta[model.select,]
238 c.coef<-c(theta,beta)
239
240 }
241 }
242
243 else if (model.select == "all") {
244
245 beta<-fit$beta
246 theta<-fit$theta
247 c.coef<-cbind(theta,beta)
248 colnames(c.coef)<- c("intercept",colnames(fit$w)[-1],colnames(fit$x))
249 rownames(c.coef)<-as.character(1:dim(beta)[1])
250 }
251
252
253
254 output<-list(coef=c.coef)
255 output
256 }
257
```

```
##############################
#summary function#########
##############################

summary.poisson.gmifs<- function(fit, model.select=NA) {
#browser()
if (is.na(model.select)) {
model.select=dim(fit$beta)[1]
Likelihood<-fit$Likelihood[model.select]
AIC<-fit$AIC[model.select]
summary<-c(Likelihood,AIC)
names(summary)<- c("Likelihood","AIC")
}
else if (model.select == "AIC") {
       model.select = fit$model.select
       Likelihood<-fit$Likelihood[model.select]
       AIC<-fit$AIC[model.select]
       summary<-c(Likelihood,AIC)
       names(summary)<- c("Likelihood","AIC")
}
else if (model.select=="all") {
Likelihood<-fit$Likelihood
AIC<-fit$AIC
summary<-cbind(Likelihood,AIC)
colnames(summary)<- c("Likelihood","AIC")
}

```

```
285 else {
286 Likelihood<-fit$Likelihood[model.select]
287 AIC<-fit$AIC[model.select]
288 summary<-cbind(Likelihood,AIC)
289 colnames(summary)<- c("Likelihood","AIC")
290 }
291
292 output<-list(summary=summary)
293 output
294 }
295
296 ###############################
297 #plot function#########
298 ###############################
299
300 plot.poisson.gmifs<- function(fit, type,main=type,beta="All") {
301 #browser()
302 if (type=="coefficients") {
303 if (beta=="All"){
304 n<-which(fit$beta[dim(fit$beta)[1],] != 0)
305 plot(1:dim(fit$beta)[1],fit$beta[,n[1]],xlab="Step",ylab="Beta",main=main,
       col=500+n[1],type="l",ylim=c(min(fit$beta[dim(fit$beta)[1],]),max(fit$
       beta[dim(fit$beta)[1],])))
306 for (i in 2:length(n)){
307 lines(fit$beta[,n[i]],col=500+n[i])
308  }
309    }
```

```
310 else {
311   n<-beta
312 plot(1:dim(fit$beta)[1],fit$beta[,n[1]],xlab="Step",ylab="Beta",main=main,
       col=500+n[1],type="l",ylim=c(min(fit$beta[dim(fit$beta)[1],]),max(fit$
       beta[dim(fit$beta)[1],])))
313 for (i in 2:length(n)){
314 lines(fit$beta[,n[i]],col=500+n[i])
315 }
316 }
317 }
318 else if (type == "AIC") {
319       plot(1:length(fit$AIC),fit$AIC,xlab="Step",ylab="AIC",main=main)
320 }
321 else { type = "Likelihood"
322 plot(1:length(fit$Likelihood),fit$Likelihood,xlab="Step",ylab="-
       logLikelihood",main=main)
323 }
324
325 }
```

6.3.2 GMIFS Simulations

```
1 ##############################
2 # No offset simulations
3 ##############################
4 #create matrix to save results in
5 sims<-matrix(nrow=100,ncol=27)
6 colnames(sims)<-c("no.correct.gmifs","beta1.gmifs","beta2.gmifs","beta3.
    gmifs","beta4.gmifs","beta5.gmifs","no.incorrect.gmifs","sum.incorrect.
    gmifs","res.sqr.gmifs","no.correct.path","beta1.path","beta2.path","
    beta3.path","beta4.path","beta5.path","no.incorrect.path","sum.incorrect
    .path","res.sqr.path","no.correct.path.norm","beta1.path.norm","beta2.
    path.norm","beta3.path.norm","beta4.path.norm","beta5.path.norm","no.
    incorrect.path.norm","sum.incorrect.path.norm","res.sqr.path.norm")
7 y.sims<-matrix(nrow=30,ncol=100)
8 library(glmpath) #load glmpath library
9 #loop 100 times or some other number
10
11 for (i in 1:100) {
12   #number of samples
13   n<-30
14
15   #generate related coefficients - these can be changed
16   beta0<-1
17   beta1<-0.3
18   beta2<-0.2
19   beta3<- -0.7
20   beta4<-0.5
```

```
21  beta5<-0.1
22  beta<-cbind(beta1,beta2,beta3,beta4,beta5)
23
24  #generate covariates related to response
25  set.seed(i*23) #set seed to replicate sims
26  x1<-runif(n=n, min=0, max=1)
27  x2<-runif(n=n, min=0, max=1)
28  x3<-runif(n=n, min=0, max=1)
29  x4<-runif(n=n, min=0, max=1)
30  x5<-runif(n=n, min=0, max=1)
31
32  #generate lambda value using the five covariates related to response
33  mu<-exp(beta0 + (beta1*x1) + (beta2*x2) + (beta3*x3) + (beta4*x4) + (beta5
        *x5))
34
35
36 #generate poisson variable using mu
37 y<-rpois(n=n, lambda=mu)
38 y.sims[,i]<-y
39
40
41 #create data frame
42 x<-cbind(x1,x2,x3,x4,x5)
43 data<-data.frame(y=y,x=x)
44
45 #create 95 covariates not related to response for a total of 100
46 x.unrelated<-matrix(nrow=n, ncol=95)
```

```
for (j in 1:95) {
x.unrelated[,j]<-runif(n=n,min=0,max=1)
}


#final data frame
data<-data.frame(data,x.unrelated)


##############################
#Create test data set
##############################

#generate covariates related to response
#set seed to replicate sims
  x1.test<-runif(n=n, min=0, max=1)
  x2.test<-runif(n=n, min=0, max=1)
  x3.test<-runif(n=n, min=0, max=1)
  x4.test<-runif(n=n, min=0, max=1)
  x5.test<-runif(n=n, min=0, max=1)


  #generate lambda value using the five covariates related to response
```

```
73   mu.test<-exp(beta0 + (beta1*x1.test) + (beta2*x2.test) + (beta3*x3.test) +
         (beta4*x4.test) + (beta5*x5.test))
74
75
76 #generate poisson variable using mu
77 y.test<-rpois(n=n, lambda=mu.test)
78
79
80 #create data frame
81 x.test<-cbind(x1.test,x2.test,x3.test,x4.test,x5.test)
82 data.test<-data.frame(y.test=y.test,x.test=x.test)
83
84 #create 95 covariates not related to response for a total of 100
85 x.unrelated.test<-matrix(nrow=n, ncol=95)
86 for (j in 1:95) {
87 x.unrelated.test[,j]<-runif(n=n,min=0,max=1)
88 }
89
90
91
92 #final data frame
93 data.test<-data.frame(data.test,x.unrelated.test)
94
95
96 ##############################
97 #run gmifs
98 fit<-poisson.gmifs(y~1, x=data[,-1], data=data)
```

```
pred<-exp(c(fit$theta[fit$model.select],fit$beta[fit$model.select,]) %*% t(
    cbind(fit$w,data.test[,-1]))) #get predicted values of test data

fit.path<-glmpath(x=as.matrix(data[,-1]),y=as.matrix(data[,1]),family=
    poisson,lambda2=0)
pred.path<- predict(fit.path, newx=as.matrix(data.test[,-1]), type="response
    ") #get response values using test data
coef.path<- predict(fit.path, newx=as.matrix(data[,-1]), type="coefficients"
    )
fit.path.norm<- glmpath(x=as.matrix(data[,-1]),y=as.matrix(data[,1]),family=
    gaussian)
pred.path.norm<- predict(fit.path.norm, newx=as.matrix(data.test[,-1]), type
    ="response") #get response values using test data
coef.path.norm<- predict(fit.path.norm, newx=as.matrix(data[,-1]), type="
    coefficients")

#different results to save from sims...
#keeping beta values, number nonzero that should be and number nonzero that
    should not be
no.correct<- sum(fit$beta[fit$model.select,1:5] != 0)
sims[i,1]<-no.correct
sims[i,2:6]<-fit$beta[fit$model.select,1:5]
no.incorrect<- sum(fit$beta[fit$model.select,-(1:5)] != 0)
sims[i,7]<-no.incorrect
sims[i,8]<- sum(abs(fit$beta[fit$model.select,which(fit$beta[fit$model.
    select,-(1:5)] != 0)+5]))
sims[i,9]<-sum((data.test[,1] - pred)^2)
```

```
####
no.correct.path<- sum(coef.path[which.min(summary(fit.path)$AIC),2:6] != 0)
sims[i,10]<-no.correct.path
sims[i,11:15]<-coef.path[which.min(summary(fit.path)$AIC),2:6]
no.incorrect.path<-sum(coef.path[which.min(summary(fit.path)$AIC),-(1:6)] !=
     0)
sims[i,16]<-no.incorrect.path
sims[i,17]<- sum(abs(coef.path[which.min(summary(fit.path)$AIC),which(coef.
    path[which.min(summary(fit.path)$AIC),-(1:6)] != 0)+6]))
sims[i,18]<-sum((data.test[,1] - pred.path[,which.min(summary(fit.path)$AIC)
    ])^2)
####
no.correct.path.norm<- sum(coef.path.norm[which.min(summary(fit.path.norm)$
    AIC),2:6] != 0)
sims[i,19]<-no.correct.path.norm
sims[i,20:24]<-coef.path.norm[which.min(summary(fit.path.norm)$AIC),2:6]
no.incorrect.path.norm<-sum(coef.path.norm[which.min(summary(fit.path.norm)$
    AIC),-(1:6)] != 0)
sims[i,25]<-no.incorrect.path.norm
sims[i,26]<- sum(abs(coef.path.norm[which.min(summary(fit.path.norm)$AIC),
    which(coef.path.norm[which.min(summary(fit.path.norm)$AIC),-(1:6)] != 0)
    +6]))
sims[i,27]<-sum((data.test[,1] - pred.path.norm[,which.min(summary(fit.path.
    norm)$AIC)])^2)
print(i)
}

```

```
save(sims,y.sims,file="simsnoffset30independent.RData")


#####################################
#Sims using an offset #
#####################################
sims<-matrix(nrow=100,ncol=27)
colnames(sims)<-c("no.correct.gmifs","beta1.gmifs","beta2.gmifs","beta3.
    gmifs","beta4.gmifs","beta5.gmifs","no.incorrect.gmifs","sum.incorrect.
    gmifs","res.sqr.gmifs","no.correct.path","beta1.path","beta2.path","
    beta3.path","beta4.path","beta5.path","no.incorrect.path","sum.incorrect
    .path","res.sqr.path","no.correct.path.norm","beta1.path.norm","beta2.
    path.norm","beta3.path.norm","beta4.path.norm","beta5.path.norm","no.
    incorrect.path.norm","sum.incorrect.path.norm","res.sqr.path.norm")
y.sims<-matrix(nrow=30,ncol=100)
library(glmpath) #load glmpath library
#loop 100 times or some other number
for (i in 1:100) {
#number of samples
n<-30
set.seed(i*34)
#generate related coefficients - these can be changed
beta0<--7
beta1<-0.3
beta2<-0.2
beta3<- -0.7
```

```
beta4<-0.5
beta5<-0.1
beta<-cbind(beta1,beta2,beta3,beta4,beta5)

#generate covariates related to response
x1<-runif(n=n, min=0, max=1)
x2<-runif(n=n, min=0, max=1)
x3<-runif(n=n, min=0, max=1)
x4<-runif(n=n, min=0, max=1)
x5<-runif(n=n, min=0, max=1)

#Generate offset
offset<- round(2000 + runif(n=n,min=-200,max=200))

#generate lambda value using the five covariates related to response
mu<-exp(beta0 + log(offset) + (beta1*x1) + (beta2*x2) + (beta3*x3) + (beta4*
    x4) + (beta5*x5))


#generate poisson variable using mu
y<-rpois(n=n, lambda=mu)
y.sims[,i]<-y


#create data frame
x<-cbind(x1,x2,x3,x4,x5)
data<-data.frame(y=y,x=x)
```

```
183
184 #create 95 covariates not related to response for a total of 100
185 x.unrelated<-matrix(nrow=n, ncol=95)
186 for (j in 1:95) {
187 x.unrelated[,j]<-runif(n=n,min=0,max=1)
188 }
189
190
191
192 #final data frame
193 data<-data.frame(data,x.unrelated,offset=offset)
194
195
196 ######################################
197 #Generate test data set
198 ######################################
199
200 #generate covariates related to response
201 x1.test<-runif(n=n, min=0, max=1)
202 x2.test<-runif(n=n, min=0, max=1)
203 x3.test<-runif(n=n, min=0, max=1)
204 x4.test<-runif(n=n, min=0, max=1)
205 x5.test<-runif(n=n, min=0, max=1)
206
207
208
209 #Generate offset
```

```
offset.test<- round(2000 + runif(n=n,min=-200,max=200))

#generate lambda value using the five covariates related to response
mu.test<-exp(beta0 + log(offset.test) + (beta1*x1.test) + (beta2*x2.test) +
      (beta3*x3.test) + (beta4*x4.test) + (beta5*x5.test))


#generate poisson variable using mu
y.test<-rpois(n=n, lambda=mu.test)


#create data frame
x.test<-cbind(x1.test,x2.test,x3.test,x4.test,x5.test)
data.test<-data.frame(y.test=y.test,x.test=x.test)

#create 95 covariates not related to response for a total of 100
x.unrelated.test<-matrix(nrow=n, ncol=95)
for (j in 1:95) {
x.unrelated.test[,j]<-runif(n=n,min=0,max=1)
}


#final test data frame
data.test<-data.frame(data.test,x.unrelated.test,offset.test=offset.test)

#test gmifs
```

```
fit<-poisson.gmifs(y~1, x=data[,-c(1,102)], data=data,offset=log(offset))
offset.test<-data.test[,102]
pred<-exp(c(fit$theta[fit$model.select],1,fit$beta[fit$model.select,]) %*% t
    (cbind(fit$w,log(offset.test),data.test[,-c(1,102)])))
offset<-fit$offset

#run glmpath
fit.path<-glmpath(x=as.matrix(data[,-c(1,102)]),y=as.matrix(data[,1]),offset
    =offset,family=poisson,lambda2=0)
pred.path<- predict(fit.path, newx=as.matrix(data.test[,-c(1,102)]),offset=
    offset.test, type="response")
coef.path<- predict(fit.path, newx=as.matrix(data[,-c(1,102)]),offset=offset
    , type="coefficients")
fit.path.norm<- glmpath(x=as.matrix(data[,-c(1,102)]),y=as.matrix(data[,1]),
    family=gaussian,offset=offset)
pred.path.norm<- predict(fit.path.norm, newx=as.matrix(data.test[,-c(1,102)
    ]),offset=offset.test, type="response")
coef.path.norm<- predict(fit.path.norm, newx=as.matrix(data[,-c(1,102)]),
    offset=offset, type="coefficients")

#different results to save from sims...
#keeping beta values, number nonzero that should be and number nonzero that
    should not be
no.correct<- sum(fit$beta[fit$model.select,1:5] != 0)
sims[i,1]<-no.correct
sims[i,2:6]<-fit$beta[fit$model.select,1:5]
no.incorrect<- sum(fit$beta[fit$model.select,-(1:5)] != 0)
```

```
sims[i,7]<-no.incorrect
sims[i,8]<- sum(abs(fit$beta[fit$model.select,which(fit$beta[fit$model.
    select,-(1:5)] != 0)+5]))
sims[i,9]<-sum((data[,1] - pred)^2)
####
no.correct.path<- sum(coef.path[which.min(summary(fit.path)$AIC),2:6] != 0)
sims[i,10]<-no.correct.path
sims[i,11:15]<-coef.path[which.min(summary(fit.path)$AIC),2:6]
no.incorrect.path<-sum(coef.path[which.min(summary(fit.path)$AIC),-(1:6)] !=
     0)
sims[i,16]<-no.incorrect.path
sims[i,17]<- sum(abs(coef.path[which.min(summary(fit.path)$AIC),which(coef.
    path[which.min(summary(fit.path)$AIC),-(1:6)] != 0)+6]))
sims[i,18]<-sum((data.test[,1] - pred.path[,which.min(summary(fit.path)$AIC)
    ])^2)
####
no.correct.path.norm<- sum(coef.path.norm[which.min(summary(fit.path.norm)$
    AIC),2:6] != 0)
sims[i,19]<-no.correct.path.norm
sims[i,20:24]<-coef.path.norm[which.min(summary(fit.path.norm)$AIC),2:6]
no.incorrect.path.norm<-sum(coef.path.norm[which.min(summary(fit.path.norm)$
    AIC),-(1:6)] != 0)
sims[i,25]<-no.incorrect.path.norm
sims[i,26]<- sum(abs(coef.path.norm[which.min(summary(fit.path.norm)$AIC),
    which(coef.path.norm[which.min(summary(fit.path.norm)$AIC),-(1:6)] != 0)
    +6]))
```

```
273 sims[i,27]<-sum((data.test[,1] - pred.path.norm[,which.min(summary(fit.path.
       norm)$AIC)])^2)
274 print(i)
275 }
276
277 save.image("simsoffset30alphaindependent.RData")
```

6.3.3 Gene Expression Code

```
1 ##############################################
2 #Set up Gene Expression Data
3 ##############################################
4 library("GEOquery")
5 data<-getGEO("GSE31836")[[1]]
6 setwd("C:/Users/makowski/Desktop/Dissertation/TCRdata/micronuclei/codes")
7 mn.data<-read.csv("MNdataCEBPpaper.csv")
8 pData(data)$Array<-substr(pData(data)$supplementary_file,93,98)
9 mn.data$ID<-paste(mn.data$Array,mn.data$Lane,sep="")
10 names(mn.data)[2]<-"Chip"
11 pheno.frame<-merge(pData(data),mn.data,by.x="Array",by.y="ID",all.x=TRUE,all
     .y=TRUE)
12 pheno.sorted<-pheno.frame[match(pData(data)$geo_accession,pheno.frame$geo_
     accession),]
13 pData(data)$C_NMNCB_VUB<-pheno.sorted$C_NMNCB_VUB
14 pData(data)$Chip<-pheno.sorted$Chip
15 pData(data)$Lane<-pheno.sorted$Lane
16 mn.exprs.data<-data[,!is.na(pData(data)$C_NMNCB_VUB)]
17 hist(pData(data)$C_NMNCB_VUB)
18 pData(data)$characteristics_ch2.1 # gender
19 nas<-apply(exprs(mn.exprs.data),1,function(x) sum(is.na(x)))
20 mn.exprs<-mn.exprs.data[nas==0,]
21 mn.gender<-pData(data)$characteristics_ch2.1[which(pData(data)$C_NMNCB_VUB!=
     "NA")]
22 all.equal(as.character(pData(mn.exprs)$geo_accession),sampleNames(mn.exprs))
23
```

```
24 # Fit model where pData(mn.exprs)$C_NMNCB_VUB ~pData(data)$characteristics_
      ch2.1(not penalized) + expression (penalized)
25
26 gexpr<-t(exprs(mn.exprs))
27 mn.gender.num<-rep(0,29)
28 mn.gender.num[grep("female",mn.gender)]=1 #male=0 female=1
29 pheno<-as.data.frame(cbind(pData(mn.exprs)$C_NMNCB_VUB,mn.gender.num))
30 colnames(pheno)<-c("MN","gender")
31
32
33
34 GErun<-poisson.gmifs(MN~gender, data=pheno, x=gexpr, epsilon=0.001, tol=1e
      -5, scale=TRUE, verbose=TRUE) #GMIFS run
35
36 pred<-exp(c(GErun$theta[GErun$model.select,],GErun$beta[GErun$model.select
      ,]) %*% t(cbind(GErun$w,scale(GErun$x,center=TRUE,scale=TRUE)))) #get
      the predicted values from GMIFS at AIC value
37 sum(coef.poisson.gmifs(GErun,model.select="AIC")$coef != 0) #how many are
      non-zero using function
38 summary.poisson.gmifs(GErun) #test of function
39 plot.poisson.gmifs(GErun,type="coefficients") #test of plot function
40
41 save(GErun,file="GErun.RData")
42
43
44
45
```

```
################################
### GLMpath run #########
################################

library(glmpath)
x<- gexpr
y<-pheno[,1]
x.nopenalty<-cbind(pheno[,2],x)

fit.path<-glmpath(x=as.matrix(x.nopenalty),y=y,nopenalty.subset=c(1),family=
    poisson,lambda2=0) #glmpath run

save.image("GErun.RData")

coef.path<- predict(fit.path, newx=as.matrix(x.nopenalty), type="
    coefficients") #get the ceofficients from glmpath
hist(coef.path[which.min(summary(fit.path)$AIC),]) #look at histogram
sum(coef.path[which.min(summary(fit.path)$AIC),] != 0) #non-zero at AIC
    value
sum(coef.path[dim(coef.path)[1],] != 0) #non-zero at full model

 ############################
 # Comparison
 ############################

 glmcoef<-which(coef.path[which.min(summary(fit.path)$AIC),] != 0) #23 non-
     zero ceofficients
```

```
69 poisscoef<-which(coef.poisson.gmifs(GErun,model.select="AIC")$coef != 0) #
       17 non-zero ceofficients
70
71 length(intersect(glmcoef[-c(1,2)],poisscoef[-c(1,2)])) #10 of them are in
      common
72
73 glmpathnon<-names(coef.path[which.min(summary(fit.path)$AIC),])[glmcoef[-c
      (1,2)]] #get the names of the glmpath
74 gmifsnon<-names(coef.poisson.gmifs(GErun,model.select="AIC")$coef[poisscoef
      [-c(1,2)]])
75
76 common<-intersect(names(coef.path[which.min(summary(fit.path)$AIC),])[
      glmcoef[-c(1,2)]], names(coef.poisson.gmifs(GErun,model.select="AIC")$
      coef[poisscoef[-c(1,2)]]))
77
78 union(names(coef.path[which.min(summary(fit.path)$AIC),])[glmcoef[-c(1,2)]],
       names(coef.poisson.gmifs(GErun,model.select="AIC")$coef[poisscoef[-c
      (1,2)]]))
79
80 outersect <- function(x, y, ...) {
81   big.vec <- c(x, y, ...)
82   duplicates <- big.vec[duplicated(big.vec)]
83   setdiff(big.vec, unique(duplicates))
84 }
85
86 gmifsonly<-outersect(gmifsnon,common)
87 glmpathonly<-outersect(glmpathnon,common)
```

```
88
89 ############################
90 #prediction
91 ############################
92
93 pred.path<- predict(fit.path, newx=as.matrix(x.nopenalty), type="response")
94
95 pred.path[,which.min(summary(fit.path)$AIC)]
96
97 png(file = "glmpathplot.png", width = 1080, height = 768, units = "px")
98 par(mar = c(10,10,1,1))
99 plot(y,pred.path[,which.min(summary(fit.path)$AIC)],xlab="",ylab="",cex.axis
       = 1.5,xlim=c(0,25))
100 myylab="Predicted MN count"
101 myxlab="Actual MN count"
102 mtext(myylab, side = 2, line = 7, cex = 3)
103 mtext(myxlab, side = 1, line = 7, cex = 3)
104 abline(lm(pred.path[,which.min(summary(fit.path)$AIC)]~y)$coef)
105 dev.off()
106
107 png(file = "gmifsplot.png", width = 1080, height = 768, units = "px")
108 par(mar = c(10,10,1,1))
109 plot(y,pred,xlab="",ylab="",cex.axis = 1.5,xlim=c(0,25))
110 myylab="Predicted MN count"
111 myxlab="Actual MN count"
112 mtext(myylab, side = 2, line = 7, cex = 3)
113 mtext(myxlab, side = 1, line = 7, cex = 3)
```

```
114 abline(lm(pred[1,]~y)$coef)
115 dev.off()
116
117 png(file = "GEMNhist.png", width = 1200, height = 768, units = "px")
118 par(mar = c(8,7,4,2) + 0.1)
119 hist(y,main="",xlab="MN Frequency",ylab="Number of Subjects",cex.lab=2,cex.
       main=2,cex.axis=2)
120 dev.off()
121
122 SSR.glmpath=sum((y-pred.path[,which.min(summary(fit.path)$AIC)])^2)
123 SSR.GMIFS=sum((y-pred[1,])^2)
```

6.3.4 Buds Code

```
setwd("C:/Users/makowski/Desktop/Dissertation/TCRdata/micronuclei")
load("BreastCa.RData")

age<-phenoData$age
smoking.status<-phenoData$CurrentlySmoking
MNfreq<-apply(phenoData[,grep("mn_Bi_w_mn",names(phenoData))], 1, sum)+apply
    (phenoData[,grep("mn_MN",names(phenoData))], 1, sum)
BNcells<-apply(phenoData[,grep("mn_Binucleate",names(phenoData))], 1, sum) +
     MNfreq
head(phenoData[,grep("mn_Mononucleate_w_MN", names(phenoData))])
Mono.cells<-apply(phenoData[,grep("mn_Mononucleate_w_MN",names(phenoData))],
     1, sum)
Tri.cells<-apply(phenoData[,grep("mn_Trinucleate",names(phenoData))], 1, sum
    ) + apply(phenoData[,grep("mn_tri_w_mn",names(phenoData))], 1, sum)
Total.cells<-BNcells+Mono.cells+Tri.cells
Bud.cells<-apply(phenoData[,grep("mn_Buds",names(phenoData))], 1, sum)
Bridge.cells<-apply(phenoData[,grep("mn_Bridges",names(phenoData))], 1, sum)
Total.cells<-BNcells+Mono.cells+Tri.cells+Bud.cells+Bridge.cells

#check distribution of Bud and Bridge counts
#both look skewed but Bridge.cells has 54/73 0 values whereas Bud.cells only
     has 16/73 0 values...run analysis on Buds
hist(Bud.cells)
hist(Bridge.cells)

NAs<-which(is.na(Bud.cells))
```

```
22
23 #filter out CpG sites that are all above 80% or below 20%
24 x.eighty<-matrix(nrow=dim(beta.corrected)[1],ncol=1)
25 for (i in 1:dim(beta.corrected)[1]){
26 x.eighty[i,1]<-sum(beta.corrected[i,] > 0.8)
27 }
28 x.eighty.pos<-which(x.eighty[,1]==73)
29
30 x.twenty<-matrix(nrow=dim(beta.corrected)[1],ncol=1)
31 for (i in 1:dim(beta.corrected)[1]){
32 x.twenty[i,1]<-sum(beta.corrected[i,] < 0.2)
33 }
34 x.twenty.pos<-which(x.twenty[,1]==73)
35
36 x.twenty.eighty.pos<-c(x.twenty.pos,x.eighty.pos)
37
38 methyl.filter<-t(beta.corrected[-(x.twenty.eighty.pos),])
39
40 #Remove observations with NA's... 70 observations now
41 Bud.cells<-Bud.cells[-NAs]
42 age<-age[-NAs]
43 smoking.status<-smoking.status[-NAs]
44 BNcells<-BNcells[-NAs]
45 methyl.filter<-methyl.filter[-NAs,]
46
47
```

```
48 ## Identify CpG sites associated with Buds for (1) general conclusions and
      (2) use for filtering in multivariable modeling step
49 pvalue<-numeric()
50 for (i in 1:dim(methyl.filter)[2]) {
51         fit<-glm(Bud.cells ~ age + smoking.status + methyl.filter[,i] +
              offset(log(BNcells)), family=poisson)
52         summary.fit<-summary(fit)
53
54        pvalue[i]<-summary.fit$coefficients[4, "Pr(>|z|)"]
55        print(i)
56 }
57
58 pvalue<-cbind(pvalue,colnames(methyl.filter))
59 colnames(pvalue)<-c("p-value","CpG")
60
61
62
63
64 ## Use a generous p-value threshold to filter CpG sites are associated with
      Buds
65 pval<-(as.numeric(pvalue[,1]))
66 sum(pval<0.05) #leaves 10860 CpG sites;
67 sum(pval<0.01) #would reduce to 1728
68
69
70 methyl.filter.pval<-methyl.filter[,which(pval < .05)]
71
```

```
smoke<-rep(1,70)
smoke[which(smoking.status == "No")] <- 0

pheno<-data.frame(age=age, smoke=smoke,binucleate_total=BNcells,binucleate_
    Buds=Bud.cells)

#save image that has everything needed to do the test run including GMIFS
    function
save.image("filterBudsrun.RData")

#GMIFS run using Buds
system.time(Budsrun<-poisson.gmifs(binucleate_Buds~age + smoke, data=pheno,
    x=methyl.filter.pval, offset=log(pheno$binucleate_total), epsilon=0.001,
     tol=1e-5, scale=TRUE, verbose=TRUE))
# user system elapsed
#12858.81 1689.74 14582.69

pred<-exp(c(Budsrun$theta[Budsrun$model.select,],1,Budsrun$beta[Budsrun$
    model.select,]) %*% t(cbind(Budsrun$w,Budsrun$offset,scale(Budsrun$x,
    center=TRUE,scale=TRUE)))) #get the predicted values from GMIFS at AIC
    value
sum(coef.poisson.gmifs(Budsrun,model.select="AIC")$coef != 0) #how many are
    non-zero using function
coef.poisson.gmifs(Budsrun,model.select="AIC")$coef[which(coef.poisson.gmifs
    (Budsrun,model.select="AIC")$coef != 0)]


```

```
######################
#plots
########################

png(file = "gmifsplotbuds.png", width = 1080, height = 768, units = "px")
par(mar = c(10,10,1,1))
plot(y,pred,xlab="",ylab="",cex.axis = 1.5,xlim=c(0,7))
myylab="Predicted Bud count"
myxlab="Actual Bud count"
mtext(myylab, side = 2, line = 7, cex = 3)
mtext(myxlab, side = 1, line = 7, cex = 3)
abline(lm(pred[1,]~y)$coef)
dev.off()

png(file = "Budshist.png", width = 1200, height = 768, units = "px")
par(mar = c(8,7,4,2) + 0.1)
hist(y,main="",xlab="Buds Frequency",ylab="Number of Subjects",cex.lab=2,cex
    .main=2,cex.axis=2)
dev.off()
```

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Academic Professional Experience

- Statistical Consultant, Technology Services, VCU 2010-2014
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- Research Assistant, Dr. Mark Reimers,Department of Biostatistics, VCU 2010-2012

- Fellow, Biotherapy Section, Laboratory of Molecular Biology, National Cancer Institute, NIH 2007-2009
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- 2007 Intramural Research Training Award, National Institutes of Health
- 2003 John Dickinson Scholar, Dickinson College

Presentations

- Sarnovsky R, Rea J, Makowski M, Hertle R, Kelly C, Antignani A, Pastrana DV, Fitzgerald DJ. Activation of Aminopeptidase from Pseudomonas aeruginosa. National Cancer Institute, Bethesda, MD 20892, July 2006.
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